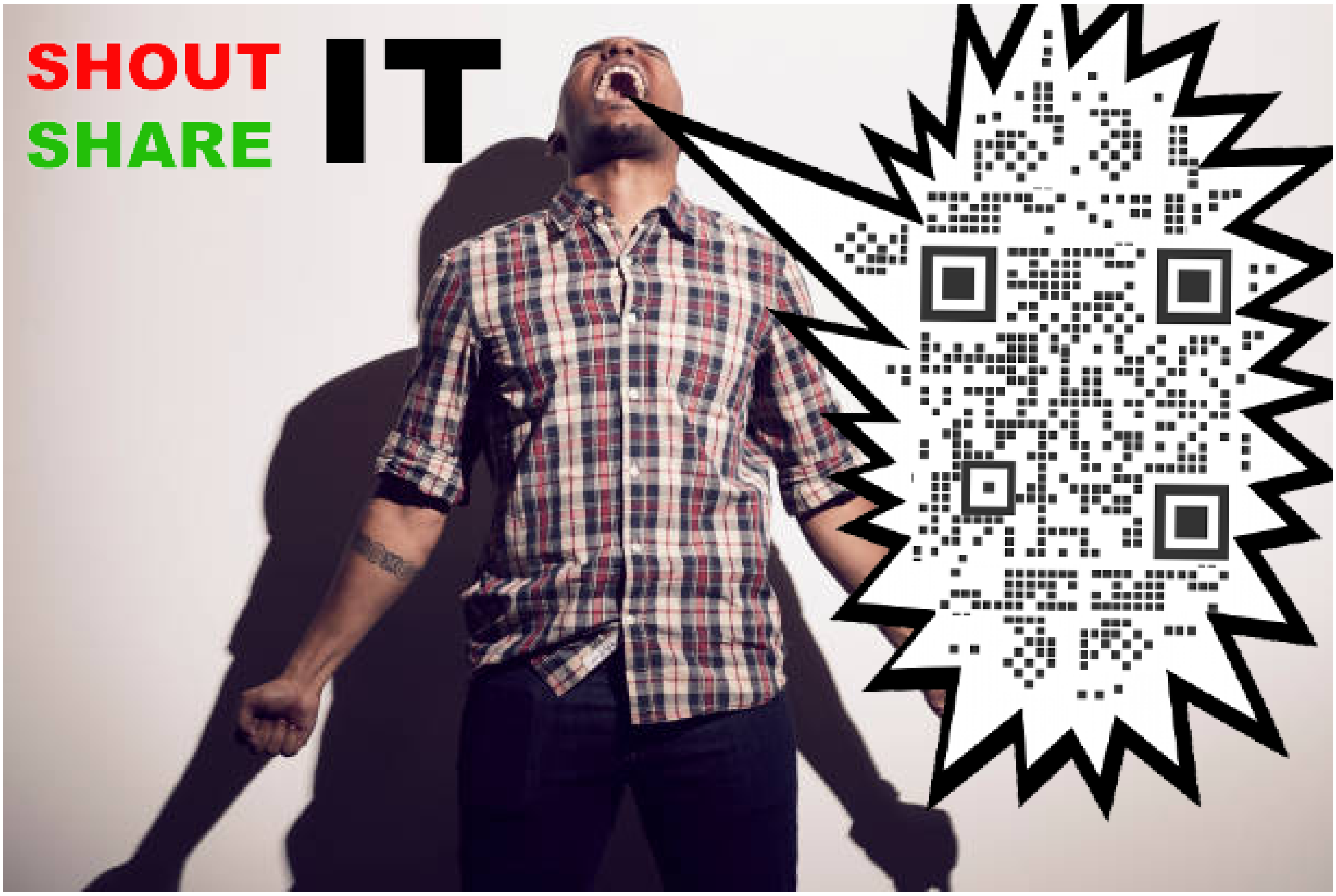
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THE METABOLIC GRADIENTS OF VERTEBRATE EMBRYOS. III. THE CHICK.

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The aim of these investigations was stated in the preceding papers of this series (Hyman, '21, '26) and repetition here seems unnecessary. Briefly it is hoped that the facts presented in these papers may serve as a basis for the interpretation of normal and teratological development. The method consists in observing the time of death of different regions of the embryo in lethal solutions. The time elapsing between exposure to the solution and death is taken as roughly proportional to metabolic rate and the method thus serves to reveal differences in rate of activity in different parts of the embryo. The grounds on which this interpretion is based are briefly presented in the preceding paper of this series (Hyman, '26) and have been fully discussed in various publications from this laboratory (*e.g.*, Child, '13; Hyman, '20).

The main facts about the gradients of the chick embryo have been known to me for about ten years. The gradients were worked out in detail in 1920 but the data upon the heart were not as complete as desired and publication has been delayed on this account. Every spring and fall since 1923 some time has been spent in studying the disintegration of the heart, a structure not very amenable to investigation by the susceptibility method. Much time and material must be wasted to obtain certain developmental stages which endure but an hour or two. I have finally, however, been able to bring the study to a reasonably satisfactory conclusion.

I. GENERAL DISINTEGRATION GRADIENTS IN THE CHICK.

In this section of the paper the disintegration gradients of all observable parts of the chick embryo except the heart will be described. The heart will be treated in a separate paper. The observations deal almost wholly with ectodermal and mesodermal structures as entodermal structures cannot be seen clearly enough to enable one to determine the time or course of death in them.

1. Method.—The yolks were poured out into physiological salt solution (0.9 per cent.) warmed to 40° C., and the embryo removed as usual to a watchglass. The salt solution was then withdrawn whereupon the blastoderm with the embryo flattens out upon the bottom of the watchglass. An oval ring, its central opening about the size of the area pellucida, was then cut out of hard filter paper, and lowered on the blastoderm, so that the embryo occupied the center of the opening. By this simple method the blastoderm is held flattened. The watchglass was then filled with the desired killing agent made up in 0.9 per cent. salt solution warmed to 40° C. In most cases the whole was then covered with a thin circular piece of glass with the exclusion of air bubbles. In other cases the watchglass was left uncovered. The death of the embryo was then watched under the low power of the compound microscope. The preparation was kept warm by placing it from time to time on a heated object.

Special care is necessary in removing very young embryonic stages from the yolk, for the blastoderm tears easily and further both the albumen and yolk are strongly adherent to the blastoderm. It was found necessary to proceed as follows in such cases. After emptying the yolk under salt solution, the albumen was completely dissected off leaving the yolk intact. A cut was then made around the blastoderm and by manipulating the cut edge the vitelline membrane was picked up and peeled off leaving the blastoderm still on the yolk. The point of a forceps was then passed beneath the blastoderm freeing it from the yolk. The blastoderm could then be floated off into a watchglass. The blastoderm is freed from adherent yolk by rocking it in several changes of salt solution.

The killing agents used were potassium cyanide, ammonium hydroxide, and sodium hydroxide. These three reagents were selected because of their high disintegrating powers, probably due to their alkalinity. Owing to the diaphanous nature of the chick embryo, particularly in early stages, the occurrence of disintegration changes cannot be detected unless they are well marked, hence the necessity for agents of the above character. It is also necessary to employ these agents in rather strong concentration so that death will occur in a relatively short time, for the more slowly the death changes take place the less detectable are they. Potassium cyanide was used in about 1/50 mol. concentration, dissolved in 0.9 per cent. salt solution. The ammonium hydroxide was made by adding two or three drops of strong ammonia to 50 to 100 cc. of 0.9 per cent. salt solution. A 1 per cent. solution of sodium hydroxide was prepared in 0.9 per cent. salt and several drops of this were added to the watchglass containing the embryo and half filled with salt solution. In some cases embryos were stained with neutral red before applying the sodium hydroxide; this is a very convenient procedure as the death of any part of the embryo is evidenced by a change of color in the dye from red to yellow.

The results in all three reagents were identical. Potassium cyanide and ammonia are believed to penetrate organisms readily but sodium hydroxide does not penetrate until death has occurred. Death differences cannot therefore be ascribed primarily to differences in rate of penetration of the agents for agents which enter readily and those which do not enter during life kill in the same differential manner. Further, since the agents were made up in salt solution, isotonic with the fluids of the chick, the contention of Wilson ('25) that the disintegration of animals in toxic solutions is due to the hypotonicity of such solutions cannot apply here. The disintegration of chick embryos in such isotonic solutions presents no noticeable differences from the disintegration changes of *Planaria*, for instance, in hypotonic toxic solutions.

Unless specifically stated otherwise several embryos of each stage have been observed. The death gradients in different individuals of the same stage of development are always similar with such variations as are stated in the following descriptions.

The drawings illustrating the death of chick embryos have been simplified and diagrammatized as much as possible. They are made from free-hand sketches drawn while watching the disintegration. Close stippling or solid outlines represent intact structures; open stippling dead and disintegrated structures.

2. Stages before the Appearance of the Typical Primitive Streak.—Technical difficulties have rendered it impossible to reach any satisfactory conclusions concerning very early stages. It is exceedingly difficult, practically impossible, to remove such early blastoderms without inflicting some injury upon them. Since every injury becomes the site of increased susceptibility and since injured places cannot be detected with certainty owing to the delicate texture of the blastoderm, disintegration changes observed in such early blastoderms cannot be regarded as conclusive evidence of innate metabolic differences.

Dr. Leigh Hoadley kindly dissected off several early blastoderms (seven to ten hours incubation) for me but in no case were we convinced that they had been removed uninjured. In such early stages there is generally visible a central area which is slightly more opaque than the remainder of the germinal disk. This opaque area is designated by Hoadley ('26*a*) as the "broad primitive streak"; its appearance is well shown in his figures. It is a somewhat cone-shaped thickening extending from the center to the posterior margin of the blastoderm. In all cases in which disintegration changes were clearly seen in these early blastoderms this opaque region was decidedly more susceptible than the remainder of the blastoderm and there was some evidence that the death changes progressed in it from its anterior to its posterior end.

3. Stage of the Typical Primitive Streak.—This was the earliest stage on which conclusive observations were made. It about corresponds to Fig. 44 *A*, p. 87, of Lillie's text.[1] At this time (about twelve to fifteen hours of incubation), the area pellucida is pear-shaped and the primitive streak is an elongated opaque thickening extending from near or anterior to the center of the blastoderm to its posterior margin. The disintegration of the primitive streak is illustrated in Figs. 1 to 3. Death changes

[1] Lillie, "Development of the Chick," 2d edition, 1919, Henry Holt & Co.

begin at the anterior end of the streak and progress posteriorly, rapidly at first, then more slowly. About seven embryos of this stage were observed and the course of disintegration was identical in all of them.

4. Stage of the Head Process.—This stage is illustrated in Lillie, Fig. 44 *B*, p. 87; its disintegration is shown in my Figs. 4 to 6. Disintegration begins at the anterior tip of the head process and proceeds posteriorly, at first very rapidly, indicating that there is little difference metabolically along the length of the head process, then more slowly. But one blastoderm of this stage was observed although several attempts were made to secure more of them. It seems that this stage endures but a very short time.

5. Stage of the Medullary Plate and the Head Fold.—This stage, illustrated in Figs. 7 to 10, about corresponds to Lillie's Fig. 44 *C*, p. 87. Disintegration begins at the anterior end of the primitive streak, at a point which is probably the primitive knot, and progresses posteriorly along the primitive streak as shown in Figs. 7 to 9. Disintegration is then initiated at the anterior end of the medullary plate and proceeds posteriorly as shown in Figs. 9 and 10.

This stage marks the appearance of the "double gradient" which has been shown to be characteristic of segmented animals (Hyman, '16, '21; Bellamy, '19). There are two regions of high susceptibility, the anterior end of the primitive streak and the anterior end of the neural axis.

6. Early Neural Fold Stage.—The disintegration at this time is similar to the preceding and is illustrated in Figs. 11 to 13.

7. Appearance of the First Somites.—An embryo with one somite and two intersomitic furrows is illustrated in Figs. 14 to 17. It is similar to Lillie's Fig. 50, p. 96. Disintegration begins as before at what I shall call the anterior end of the primitive streak, *i.e.*, the place where the primitive streak is differentiating into notochord and somites. This place presents a club-shaped appearance. From this point disintegration proceeds in both directions, forward involving the somitic region, backward along the streak to its posterior end. Soon the anterior end of the neural folds begins to disintegrate and this progresses posteriorly

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along the folds, meeting the disintegration which is advancing forward from the somitic region. The high susceptibility of the lateral boundaries of the embryo is also noticeable.

8. Stage of Three or Four Somites.—The disintegration of a three somite embryo is shown in Figs. 18 to 20. The death changes begin as before at the anterior differentiating end of the primitive streak and proceed in both directions from this region. A region of high susceptibility is now, however, noticeable in the center of the neural folds, as shown in Fig. 18. From this place disintegration proceeds in both directions along the neural folds. The explanation of this circumstance is simple—the neural folds are in process of fusion. As is well known, this fusion does not begin at the anterior end of the neural axis but posterior to this point in a region corresponding according to Lillie, p. 99, "approximately with the region of the future mid-brain or anterior part of the hind brain." There is thus an increased activity preceding the closure of the neural folds for the neural folds meet "by the time four or five somites are formed" (Lillie, p. 98) while the increased susceptibility of this region is already evident at a three somite stage. The somites at this time disintegrate from the segmental plate forward. The lateral boundaries of the embryo are also highly susceptible; processes are evidently in progress there in connection with the separation of the embryo from the blastoderm. The disintegration of four somite embryos is the same as that of three somite stages.

9. Five to Eight Somites.—The region of high susceptibility at the place of closure of the neural folds persists for a varying length of time. Embryos with as many as seven or eight somites may still exhibit traces of it. In such later embryos this region of initial disintegration of the neural tube has moved backward into the hindbrain. Sooner or later, however, this region of high susceptibility in the hindbrain region disappears and the disintegration gradient of the brain region is again of the simple antero-posterior type. Five somite embryos may have already reached this condition. Such an embryo is illustrated in Figs. 21 to 25. Disintegration begins as before in the clavate differentiating region of the primitive streak and proceeds in both directions, anteriorly along the posterior ends of the neural folds and the segmental plate, posteriorly to the caudal end of the embryo. Disintegration next attacks the anterior end of the neural tube and progresses posteriorly meeting the other disintegration at the level of the first pair of somites. In another embryo of five somites, the region of high susceptibility in the hindbrain, correlated with the closure of the neural folds, was still present. The disintegration of the neural tube of this embryo is shown in Figs. 26 and 27; otherwise it was identical with the embryo represented in Figs. 21 to 25.

The disintegration of an embryo of seven somites, corresponding to Lillie's Fig. 59, p. 104, is illustrated in Figs. 28 to 30. Death changes begin as always in the clavate differentiating region of the primitive streak and proceed anteriorly from here along the segmental plate and posterior ends of the neural folds. They then begin in the fore-brain, in the optic evaginations, and progress posteriorly along the neural axis. Other embryos of seven and eight somites may still exhibit barely perceptible traces of heightened susceptibility in the region where the neural folds are closing.

In all embryos up to seven or eight somites the gradient in the somites is, as far as could be determined with certainty, from the segmental plate forward. It is somewhat difficult to observe the somites in these early stages owing to the fact that the neural folds as they die spread open and conceal the somites from view. In a number of cases, however, the death of the somites was observed with certainty and is, as stated, from the segmental plate anteriorly.

10. Nine to Eleven Somites.—The chief point of interest about embryos of these ages is the rapidly increasing susceptibility of the optic evaginations. The death of an embryo of eleven somites is illustrated in Figs. 31 to 35. Disintegration begins in the anterior end of the primitive streak, as usual, and simultaneously in the tips of the optic vesicles. From the first named region it progresses anteriorly along the neural folds and segmental plate. The optic vesicles disintegrate from their lateral extremities medially; disintegration then passes posteriorly along the neural tube meeting the advancing postero-anterior disintegration at about the middle of the somites. From this time on a double gradient is present in the somites; anterior and posterior somites are the most susceptible and from them death proceeds towards the middle somites.

11. Stage of Twelve Somites.—In this, the stage of the classical 33-hour chick (Lillie, Fig. 63, p. 109), a new region of high susceptibility makes its appearance, at the anterior end of the hind brain. The disintegration of a twelve somite chick is represented in Figs. 36 to 39. The high susceptibility of the optic vesicles has now died away; evidently the process of evagination is completed. Disintegration begins in the anterior end of the primitive streak and the anterior end of the neural axis and proceeds anteriorly from the former, caudally from the latter. Very soon, however, the region in the hind brain mentioned above begins to disintegrate and from this region disintegration progresses in both directions along the neural tube as shown in Fig. 37. This occurrence, at first puzzling, was readily interpreted after later stages had been studied. It was then realized that this region of high activity in the hind brain of the twelve somite chick foreshadows physiologically the process of turning of the head of the embryo which is not manifested morphologically until a slightly later stage.

12. Fourteen to Twenty Somites.—The head of the embryo now begins to turn on its left side and the stalks of the optic vesicles narrow. Both of these processes are indicated in the disintegration. The disintegration of a fifteen somite chick is shown in Figs. 40 to 44. Death begins in the anterior end of the primitive streak and in the stalks of the optic vesicles. From the former it progresses forwards along the neural folds and segmental plate; from the latter laterally to the tips of the vesicles. Disintegration next occurs at the anterior end of the forebrain and in the hind brain; from these two regions it progresses backward and forward respectively, meeting in the region of the midbrain. The high susceptibility of the hindbrain is evidently correlated with the turning of the head; it should further be noted that the region of the brain involved in this process has moved posteriorly as compared with the preceding stage. In the remainder of the body disintegration proceeds along the neural tube and somites from the two ends of the body towards the middle, the two waves of disintegration meeting at the middle of the somites.

From this time on through more than twenty somites the disintegration gradients are about the same. The turning of the head as well as the advancing head fold of the amnion obscure observations on the disintegration of the head. The region of high susceptibility in the hindbrain becomes more marked and involves a larger area. The advancing margin of the head fold of the amnion and also the auditory pits are regions of high susceptibility.

13. Two-Day Chick.—This stage, illustrated in Figs. 45 to 47 is marked by the high susceptibility of the eye. The formation of the lens and the optic invagination are at their height. The eye and the posterior end of the embryo are the most susceptible parts. After the eye has disintegrated, disintegration begins in the tip of the telencephalon and proceeds posteriorly along the brain. A region of high susceptibility is also still present at the place where the body is turning and from this place disintegration progresses in both directions. The auditory vesicles disintegrate before the hindbrain. There is the usual postero-anterior progress of the disintegration from the primitive streak.

14. Two- to Three-Day Chicks.—In later stages the region of high susceptibility at the bend of the embryo gradually disappears; it can still be detected at the beginning of the third day of incubation but subsequently vanishes. The susceptibility of the posterior end of the embryo also gradually diminishes. In the two-day chick the tail bud is still the most susceptible part of the embryo, but in the three-day chick, the tail bud does not begin to disintegrate until sometime after anterior structures have undergone disintegration. Of anterior structures the eye is by far the most susceptible in these later stages. Disintegration begins in the lens vesicle and then extends to the optic cup. Next the telencephalon disintegrates and this process proceeds posteriorly along the brain. The gradient of the brain is rather shallow, however. At all stages which were observed the double gradient was present but the susceptibility of the posterior end as compared with the anterior end diminishes in the later stages, as already mentioned. After disintegration has begun at the posterior end it proceeds forwards, in the neural tube first, then in the somites; the last region of the embryo to disintegrate is in general at about the level of the middle somites. The anterior somites are commonly more susceptible than the posterior ones and susceptibility in the somites decreases from both ends towards the middle. The limb buds in the three-day chick are regions of moderately high susceptibility, the anterior limb buds being more susceptible than the posterior ones. The ear vesicles are more susceptible than the adjacent region of the brain in the two-day chick but their susceptibility is decreased in the three-day chick. The visceral arches in all stages in which they could be observed with certainty were the least susceptible parts of the embryo; they appeared to disintegrate from the posterior end forwards as do the somites in early stages.

In these later stages of the chick evidences of a medio-lateral gradient were noted. If we consider a section through the middle of the body, the neural tube is the most susceptible part of the section; the medial parts of the somites disintegrate next; and the disintegration then proceeds laterally along the somites to and along the lateral plate. In two- and three-day chicks where the first somites present their lateral surfaces to the observer, owing to the turning of the trunk, it could easily be seen that the medial portions of the somites are more susceptible than their lateral portions.

15. Entodermal Structures.—The posterior part of the notochord can usually be seen more or less distinctly. This part disintegrates from the primitive streak anteriorly. The anterior part of the notochord is more or less obscured but appears to disintegrate from the diencephalon caudally. The gradient in the notochord is thus probably of the double type as in the later somites and the neural tube.

If the embryo is mounted ventral side up, the ventral layer of the blastoderm, which is of course composed of entoderm, is exposed to view but is of so delicate a texture that its time of disintegration is difficult to determine. In the posterior part of embryos up to two days of age the entoderm layer also appears to disintegrate from the posterior end forward, thus accompanying the disintegration of the neural tube. The entodermal layer of the anterior intestinal portal is always a region of high susceptibility and from the portal the disintegration extends anteriorly along the entoderm of the blastoderm. The foregut could not be distinctly observed.

16. Relative Susceptibility at Different Ages.—While this matter was not subjected to definite experimental test, the much greater susceptibility of the younger stages was so obvious that it could not escape attention. A primitive streak stage will disintegrate in a few minutes in a concentration of reagent in which a two-day chick will not undergo complete disintegration in two hours.

17. General Summary of the Gradients in Chick Embryos.—In the early stages—primitive streak and head fold stages—a simple primary antero-posterior gradient is present, disintegration beginning at the anterior end of the embryonic axis and proceeding posteriorly. At the time of the differentiation of the medullary plate and folds a double gradient appears and persists through all of the stages that were investigated (through the third day). In general this double gradient is as follows: there are two regions of high susceptibility, the differentiating region of the primitive streak, and the anterior end of the neural axis; from these two regions disintegration proceeds anteriorly and posteriorly, respectively, towards the middle of the embryo. The differentiating region of the primitive streak is the most susceptible part of the embryo up to about the third day of incubation at which time it gradually decreases in activity in correlation with the completion of the somites. The double gradient involves both ectodermal and mesodermal structures; and probably also to at least some extent, entodermal structures. In earlier stages disintegration in the mesoderm proceeds from the segmental plate forward along the somites; but after eight or nine somites have appeared, the anterior and posterior somites are both regions of high susceptibility and from them disintegration proceeds backward and forward respectively towards the middle. The neural tube is usually more susceptible than the mesoderm of the same level. There is also present a mediolateral gradient in susceptibility from the neural tube towards the lateral plate.

In addition to this general double gradient, special regions of high susceptibility appear temporarily during development. The most important of these are: all places where folds, unions, or bendings are about to occur; and the special sense organs.

In general any extensive developmental change is preceded by an increased susceptibility, that is, an increased metabolic rate in the group of cells involved. Intense metabolism thus appears to be a necessary condition for active development.

II. Application to Normal Development.

The foregoing observations may throw some light on problems of amniote development and on the physiological conditions underlying normal and teratological development. In utilizing these observations for the interpretation of development, the susceptibility differences described in the preceding section will be regarded as representing differences in the rate of cell activity, the most active regions being the most susceptible to lethal solutions.

The primitive streak is described in textbooks of embryology as an undifferentiated linear ectodermal thickening. The important researches of Hoadley ('26, *a*, *b*, *c*), however, have shown that there is some localization of developmental potentialities in blastoderms younger than the typical primitive streak stage, blastoderms of four to eight hours incubation. My observations on the typical primitive streak stage (about fifteen hours incubation) indicate that there is also present a physiological gradation along the streak. The anterior end of the streak is the seat of a high rate of activity, this rate diminishing posteriorly along the streak. My observations of course do not show that this physiological differentiation precedes the morphological localization for the investigations of Hoadley concern earlier stages than those on which I was able to observe reliable susceptibility differences. But it must be recalled that in the hen's egg the direction and position of the primary antero-posterior axis of the future chick are already established in the ovocyte, being determined by the attachment of the follicle in the ovary. In the pigeon's egg also Bartelmez ('12) showed that the main axis is determined in the ovocyte and is evidenced during the blastodisc stage through the position of certain granules. As Hoadley found a lack of localization of future structures in unincubated blastoderms or those incubated two hours it appears that it must be conceded that some physiological differences underlie the subsequent localization of organs along the axis.

The nature of the head process has been a subject of discussion. My observations indicate that the head process stage does not differ as regards metabolic conditions from the primitive streak stage and thus support the conclusion of Lillie (p. 82) that the head process is simply a further development of the anterior end of the primitive streak. The region of high metabolic activity at the anterior end of the streak develops into the head process.

In subsequent development this region retains its high rate of activity and becomes the anterior end of the neural axis. But at about the time of origin of the medullary folds, a secondary region of still higher activity appears. This region is located at the anterior end of the primitive streak (excluding the head process as part of the streak) or in what is known in textbooks of embryology as the primitive knot or Hensen's node. The origin of such a second region of intense activity when one such region is already present can from a physiological point of view be ascribed only to the inability of the latter to control more than a short length of the axis. Beyond that region of control physiological isolation occurs and the origin of a new center of activity is possible. This new center being isolated from the dominance of the original center at the anterior end of the embryonic axis behaves as isolated regions always do when they are intrinsically capable of such behavior—it proceeds to the formation of new individuals, *i.e.*, segments. The experiments of Bellamy ('19) on the frog constitute the best evidence that we have of the applicability of this interpretation, originally derived by Child from experiments on lower invertebrates, to the vertebrate embryo. Bellamy showed that in the frog embryo the distance between the two centers of high activity can be altered by the action of external factors. As the anterior center of activity of the chick embryo differentiates into nervous tissue it is able to dominate more of the axis; the secondary active region, localized in the anterior end of the primitive streak thereupon moves backwards, or more strictly speaking is transferred to more and more posterior cells, leaving incomplete zooids or segments anterior to it. Finally the anterior end dominates the posterior center of activity to such an extent that it is no longer able to carry on independent processes and segment formation thereupon ceases.

How much of the embryo arises from the primitive streak is a question of considerable interest to embryologists. The answer to the question will of course depend on what is meant by primitive streak. If following the usual usage we regard the primitive streak as that portion of the early embryonic axis including and posterior to the secondary center of activity, that is, the primitive knot, the answer can be given on *a priori* grounds from the argument in the preceding paragraph as follows: the primitive streak gives rise to all of that part of the embryo which is segmented, that is, from the first somite posteriorly. This conclusion agrees with the experimental results of Peebles ('98).

One of the most striking facts that has appeared in this investigation is the high susceptibility of each region where development is progressing rapidly. This indicates that intense metabolism is a necessary condition for active development. According to my observations such developmental processes as the closure of the neural folds, elevation of the amniotic folds, and formation of the body and brain flexures are the result primarily of cell activity and not a consequence of mechanical conditions as supposed by early embryologists. It further appears from my observations that intense cellular activity precedes such developmental processes. This was clearly the case for instance as regards the closure of the medullary folds and the turning of the embryo. The increased activity in these processes is evidenced before the morphological change is initiated.

From my studies of the metabolic gradients in the embryos of the teleosts ('21), the brook lamprey ('26) and the chick and the work of Bellamy on the frog ('19) it seems justifiable to conclude that the formation of two centers of high activity is the regular mode of development of vertebrates. Two such centers have been found in all vertebrate embryos so far studied and they are always located in the same position with respect to the future embryo—one at the anterior end of the antero-posterior axis and the other in the axis at a more posterior point. The posterior center is the dorsal lip of the blastopore in cyclostome and amphibian embryos (probably also in amphioxus), the posterior end of the embryonic axis in teleost fish, and the primitive knot, subsequently the "tail bud," in the chick. This posterior center of activity is like a growing point which growing backwards deposits the trunk of the embryo anterior to it. It is interesting to note that Assheton ('94*a*, '94*b*) long ago recognized in the embryos of the frog and the rabbit the presence of these two centers of activity and correctly identified the posterior one as the dorsal lip of the blastopore in the frog and a region about corresponding to the primitive knot of birds in the rabbit. He called these regions primary and secondary centers of cell proliferation. He stated that the primary center forms the head of the embryo anterior to the first somite, while the secondary center forms the rest of the embryo. My ideas here presented entirely coincide with these statements of Assheton, which seem to have been generally overlooked. Eycleshymer ('98) working with the amphibian embryo accepted Assheton's ideas. He showed that the primary center, the center of the animal pole, becomes the anterior end of the embryo, the dorsal lip of the blastopore the trunk. He considers these to be two regions of high activity, agreeing with Assheton. He stated: "The primary area of cell activity at the upper pole of the amphibian egg forms the basis of the cephalic end of the embryo." "The secondary area of cell activity on the blastoporic side of the egg forms the basis of the greater portion of the posterior half of the embryo." (Eycleshymer entertained the probably erroneous notion that the extreme posterior end of the amphibian embryo was formed by concrescence.) Adelmann ('22) and Kingsbury ('24) seem to agree with the conception that the formation of two centers of high activity is the regular mode of development among chordates. Adelmann's idea that these two centers arise by the splitting of one center is, I think, incorrect. It does not agree with the actual facts of observation by the susceptibility method on several types of vertebrate embryos. The posterior center arises independently of and usually at some distance from the anterior center, although in the chick the two centers are somewhat close together at first. In all cases a region previously lower in susceptibility than the anterior center begins to increase in susceptibility and generally eventually surpasses the anterior center in activity for some time. Further criticisms by Adelmann and Kingsbury of the application of susceptibility data to chordate development have been answered by Child ('25).

I also wish once more to record my disbelief in the concrescence theory of the formation of the primitive streak. I find no evidence in any of my work with vertebrate embryos of the occurrence of such a process. Neither can I accept the germ ring interpretation of chordate development as expounded for instance in Kellicott's "Chordate Development." The germ ring type of development occurs in certain meroblastic eggs and appears to be simply a method of inclosing the yolk by the blastoderm. The germ ring of the teleost embryo does not appear to be of much more importance in the formation of the embryo than the growing margin of the blastoderm in the chick embryo. The posterior growing point of teleost embryos situated in the germ ring corresponds in my opinion to the dorsal lip of the blastopore of amphibian embryos. It probably appropriates neighboring cells as does also the latter.

Textbooks of vertebrate embryology should probably be revised, emphasizing the origin of the chordate embryo from two centers of activity, a primary center which forms the anterior end of the embryo and a secondary posterior center which grows backwards and forms the remainder of the embryo. The recent beautiful experiments of Spemann and Mangold ('24) establish beyond doubt the importance of this secondary center in the production of the embryo. Whether or not a primitive streak appears in the development of a chordate seems to depend on the time relation between gastrulation and the establishment of the posterior center. If the posterior center arises somewhat tardily after gastrulation development occurs with the formation of a primitive streak; but where the center is early established a primitive streak is absent from the development since differentiation then begins at once.

Faris ('24) in a study of pigment formation in the *Amblystoma* embryo has expressed the opinion that it is necessary to distinguish between differentiation metabolism and proliferation metabolism. The former has probably a greater integrative action than the latter. This idea may be very important in the interpretation of developmental processes but cannot be evaluated at present.

The question of the origin of metabolic differences in embryonic development is naturally of prime importance but little evidence is as yet available upon this matter. In the case of the hen's egg it seems definitely established that the axis of the embryo is determined by the position of the ovocyte in the ovary, *i.e.*, by an environmental factor acting in a definite direction. Similarly it may be supposed that the metabolic conditions present at each developmental stage are determined by factors operative at preceding stages. In the last analysis environment must be the cause of metabolic differences.

III. Application to Teratological Development.

Abnormal chick embryos accidentally or experimentally produced have long been of interest to zoölogists. Many articles have been published dealing with the experimental production of abnormalities in the chick but most of them are rather old and I have not been able to obtain access to a number of them. Others give such inadequate accounts of the abnormalities found that they are of little value from the present point of view.

The interpretation of experimentally produced abnormalities in the chick on the basis of differential susceptibility will naturally follow the same lines as stated in previous publications by Child and his students on developmental modifications in other animals. Those portions of the embryo which are the most susceptible to lethal concentrations of toxic agents will be most affected and modified by non-lethal exposures of proper duration and concentration in which acclimation does not occur. These same portions will recover most readily if the concentration and duration of exposure employed permit recovery and acclimation. If the metabolic rate of the egg or early embryo is permanently depressed and slowed down by the action of external agents, parts requiring for their normal development a high metabolic rate will be completely suppressed or will be abnormal because the rate of activity necessary for their normal development cannot be attained in such depressed eggs or embryos. The various agents which can be used to affect development are regarded from this viewpoint as acting merely in a quantitative way to retard or accelerate development; and this action is differential affecting some parts of the embryo more than others since some parts have or require a higher rate of activity than other parts.

Interpretations of teratological development somewhat similar to the foregoing have not been wanting. Dareste ('91) who investigated more thoroughly than anyone else before or since the experimental production of monstrosities in the chick expressed similar ideas. He states that development is due in part to inherent tendencies and in part to external conditions. As the latter can be altered, modification of the course of development is possible. Dareste reached the following important general conclusions. (1) The same abnormalities are produced by very different conditions, there being then no necessary relation between the application of a certain condition and the appearance of certain modifications. (2) Embryos submitted to the action of identical factors do not necessarily present the same abnormalities (although these may often be similar). The reason for this is that eggs are inherently different from each other in a variety of ways. (3) The different abnormalities do not depend on the nature itself of the teratogenic agents but on the time at which they act upon the embryo, their intensity, and their duration. (4) All abnormalities consist essentially of an arrest of development of the embryo or its membranes. The organs of an embryo appear successively; they pass through a certain number of stages. An arrest of development consists in the persistence of an embryonic state ordinarily transitory. Among the abnormalities assigned by Dareste to arrest of development at a certain stage are: duality of the heart, spina bifida, absence or poor development of the area vasculosa, absence or abnormalities of the amnion and allantois, defects of the brain, head, spinal cord, and sense organs. Certain subsequent interpretations of teratological development as for instance that of Stockard ('21) do not appear to differ from or add anything to Dareste's conclusions.

The work on differential susceptibility and modification of development carried on in this laboratory by Child and his students supports Dareste's fundamental conclusions but has added one important factor to the interpretation of teratological forms. The statement of Dareste that most abnormalities are the result of retarded or arrested development is correct but needs to be carried one step farther. *Inhibition or retardation modifies the development of some parts of the embryo more than of others*, so that *certain* regions *frequently* exhibit teratological development while other regions *seldom* do so. Those regions most prone to abnormal development under the action of inhibiting agents of proper concentration and duration of exposure are those which have been shown by Child and his students to be most susceptible and most quickly killed by lethal concentrations of these agents. In brief differential inhibition of development results from differences in susceptibility of different parts of the embryo.

On the basis of the susceptibility differences described in this paper the application of external agents of a depressing nature to the chick embryo should yield the following results. Agents applied at very early stages may produce a permanent general diminution of the metabolic rate of the embryo. Such a condition will result in the absence or abnormality of those parts of the embryo requiring a high metabolic rate for their normal development, for in such retarded eggs the necessary rate of activity cannot be attained. Sufficiently drastic treatment at an early age will prevent the appearance of the embryo altogether, only the blastoderm being present; for as shown in this paper the embryonic is more susceptible than the extra-embryonic portion of the germinal disk. Less drastic treatment of early embryos up through the head process stage (fifteen to eighteen hours of incubation) should cause defects of the anterior end mainly, particularly the brain, since during this period of development there is but one center of high activity, the anterior end of the embryonic axis. Exposure to depressing agents during later stages of incubation (medullary fold through the two-day chick stage) would be expected primarily to stop or retard the posterior elongation and in addition to evoke abnormalities of the anterior

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end and special sense organs; for during this time there are two centers of high susceptibility, the anterior end of the embryo and the growing region of the primitive streak. Application of depressing agents at the proper times when such processes are occurring would tend to prevent the occurrence of unions, and the formation of folds, bends, turns, etc., and would induce abnormalities in particular structures, such as the sense organs and limb buds. It is not, however, necessary in the case of structures requiring a very high rate of activity for their normal development that the inhibiting agents be applied at the time they are developing (contrary to the opinion of Stockard, '21); for any general lowering of the metabolism of an embryo at an early stage hinders the future development of such structures. Thus it has been shown that the usual typical abnormalities appear when the egg is subjected to depressing agents before fertilization or when the sperm are injured (for literature on this matter see Hyman, '21). Probably structures which develop at a low metabolic level cannot be inhibited or altered unless depressing agents are applied at the time of their greatest developmental activity.

As noted long ago by Dareste, abnormalities can be induced in the chick by a variety of methods and such abnormalities bear no specific relation to the agent employed. I shall here review the different agents which have been used and the abnormalities obtained by their action, considering only single monsters.

Dareste ('83*b*, '91, p. 103 ff.) found that shaking eggs induces abnormalities of development. Eggs incubated immediately after a railroad journey are apt to develop abnormally but yield normal embryos if several days elapse between the journey and incubation. Eggs shaken violently on a machine yield a high percentage of abnormalities whether incubated immediately or some time after being shaken. In about half of the eggs so treated blastoderms without embryos were found. When embryos were present these were generally abnormal, exhibiting such conditions as doubled hearts, defective eyes, reduced size, defective brains, spina bifida, and absence of the posterior end.

According to Dareste and others (Dareste, '91, pp. 161, 168), eggs tend to develop abnormally if they are not turned during incubation or if they are incubated in the vertical position. Few details are, however, furnished as to the types of abnormalities so induced. They are stated to result from the adherence of the embryonic membranes to the shell.

Several workers have reported induction of teratological development in the hen's egg through exposure of the eggs to the electric current or a magnetic field (Lombardini, '68, Maggiorani, '84, Windle, '91, '93, '95). I have not been able to obtain these publications except two of Windle's but it appears that very few details were given in them of the types of abnormalities obtained. Probably the teratological action really resulted from the products of electrolysis rather than directly from the current or magnetic fields. Windle found a high percentage of malformed embryos with defective areæ vasculosæ but Maggiorani reported an excessive vascularization of the yolk sac with arrested development as a result of incubating eggs between two powerful magnets.

There has been some investigation of the action of X-rays and radium emanations on the development of the chick. Gilman and Baetjer ('04) exposed hen's eggs to X-rays ten minutes a day for four successive days and noted certain abnormalities—deformed occipital region, retarded eyes, membranes adherent to the embryos, and deformations of the limbs. Tur ('04) subjected hen's eggs continuously to radium placed on the outside of the shell during periods of incubation varying from 24 to 70 hours. After 24–28 hours of such treatment, blastoderms of normal size were found, but the area pellucida was reduced, and only the posterior part of the primitive streak was in evidence, the anterior part having been reduced to an irregular mass. After 45 to 70 hours of incubation under radium emanation blastoderms without visible embryos were obtained. In some cases the area vasculosa was present and more or less normal in appearance; in other blood and blood vessels were lacking and the area pellucida was greatly reduced. Sections of such blastoderms in the region where the embryo was to be expected revealed the presence of a flattened inactive ectoderm and a thickened proliferating entoderm showing numerous mitotic figures. Tur's results agree with my findings that the embryonic is more susceptible than the extra-embryonic portion of the blastoderm and the anterior part of the primitive streak more susceptible than its posterior part.

More numerous have been the attempts to modify the development of the chick by various chemicals. Féré ('94, '99*a*) exposed eggs to the vapors of ethyl and other alcohols before incubation, and reported the following abnormalities: blastoderms without embryos, arrest of development of the amnion, cyclopia, absence of the eyes, defective heads, atrophy of the head, spina bifida, absence of somites, duplicity of the heart. He also ('99*b*) tried the effect of exposure to the vapors of ammonia before incubation. Ammonia was found to be exceedingly toxic; one half hour exposure yielded blastoderms with embryos. After fifteen minutes exposure, defects similar to those obtained with alcohol resulted. Reese ('12) tried the effect of various narcotics on the development of the hen's egg but gives no details beyond the statement that abnormalities were produced. Stockard ('14) treated the egg with various chemicals and states as did Dareste that their action is not specific. Eggs were exposed to the vapors of alcohol and ether before or during incubation. The treated embryos tended to be small and developed more slowly than the controls. Among the defects noted were: blastoderms without embryos, small and reduced embryos with defective brains and circulatory systems, one or both eyes defective, cyclopia.

Alteration of the oxygen supply to the egg is a commonly used method of inducing teratological development. The earlier attempts in this direction consisted in covering the whole or portions of the egg with a varnish. The original purpose of such experiments seems to have been to prove the necessity of oxygen for the development of the chick. If the entire egg is varnished the embryo of course dies at a certain stage of development as shown long ago by Dareste. If the egg is partially varnished, development tends to be abnormal. Gerlach and Koch ('83) varnished the entire egg except for a circle 4.5 to 6 mm. in diameter immediately over the blastoderm. The general consequence of such procedure was a dwarfing, often extensive, of the embryo, due in my opinion to inhibition of the growing region of the primitive streak. Similar dwarfing accompanied by other abnormalities resulted when the unvarnished circle was over the anterior or posterior part of the blastoderm. Windle ('93) applied varnish to different parts of the egg. Among the abnormalities regularly obtained were: defects of the area vasculosa, absence of the embryo, deformed embryos. Here again the account is too lacking in detail for interpretation. Mitrophanow ('00) also tried the effect of partial shellacking of the egg, combining this procedure, however, with heightened temperature. Upon shellacking that half of the egg containing the anterior half of the embryo and incubating at supernormal temperature (about 45° C.) Mitrophanow noted a general inhibition of development of the anterior part of the primitive streak and of the head process with broadened and thickened posterior ends. Mitrophanow regards such posterior ends as overdeveloped but it seems to me that they may represent inhibitions. It appears that if the posterior center of activity is somewhat inhibited it tends to pile up into masses of cells instead of elongating in the posterior direction. In one case, which was in the early medullary fold stage when opened, the primitive knot region was found greatly inhibited, regions anterior and posterior to this being more or less normal. Shellacking the posterior half of the egg did not yield any definite modifications of development except some inhibition of the posterior end. Mitrophanow was of the general opinion that the posterior end of the early embryo is most susceptible to modification. The most extensive experiment on the production of abnormalities in birds by an altered gaseous environment is that of Riddle ('23). Unfortunately the data furnished by Riddle as to the modifications induced are very meager. Further the embryos were examined mostly at late stages or at hatching and many of the most abnormal embryos were thus probably missed, having died at early stages. Riddle records several dove and pigeon embryos with defective or absent eyes, small heads, and other head defects as a result of exposure to air reduced in oxygen content (11 to 13 per cent. oxygen) during about 24 hours in the first three days of incubation. Excess oxygen with or without increased carbon dioxide content yielded similar abnormalities. Riddle states that nearly all of the abnormalities arose from embryos "aged less than two days at the time treatment was begun." Byerly ('26) allowed eggs to incubate for 24 hours and then coated them with water-glass after removing the entire shell and outer shell membrane from the air chamber. Such embryos were then incubated for three days. Some of the embryos thus obtained were very abnormal, the posterior end being most affected. This agrees with my finding that at 24 hours the posterior end of the embryo is more susceptible than any other part.

Although the modifications induced by these various procedures agree with the expectations on the basis of differential susceptibility, nevertheless the applicability of this interpretation in these cases may be open to question: for there is no certainty that all parts of the embryos were equally exposed to these agents. Variations in different regions of an egg as regards thickness and porosity of the shell, viscosity and thickness of the albumen layer, permeability of the shell and egg membranes, and similar factors render it highly probable that external agents and conditions will not reach all parts of the embryo simultaneously. To mention only one condition, an altered gaseous environment obviously requires some time to affect the embryo. Obviously the principle of differential susceptibility will apply to the induced abnormalities with certainty only when the conditions or agents applied have reached all parts of the embryo equally and simultaneously. Apparently the only conditions under which these prerequisites are fulfilled are ageing of the eggs and alterations of the temperature of incubation.

Dareste ('82, '83*a*) found that if eggs are kept for some time before being submitted to incubation, development is abnormal. Typical abnormalities were: blastoderms without embryos, embryos with absent or reduced posterior ends, reduced and abnormal heads and brains, and celosomia (protrusion of the viscera due to a failure of the lateral limiting sulci to close below). It is possible of course that a beginning slow development at room temperature as well as senescence of the embryo is a factor in these cases.

A number of experimenters have tested the effect of subnormal or supernormal incubation temperatures on the development of the chick. Dareste regarded alteration of temperature as one of the best methods of inducing teratological development in the chick. According to Dareste, the hen's egg will not develop below 28° C. but Edwards ('02) places the "developmental zero" at 20°–21° C. Dareste found that the embryo dies at 44° or above but Mitrophanow ('00) would seem to have obtained development at temperatures up to 50. According to Dareste development below 35° or above 39° C. is nearly always abnormal.

Féré ('94) in eggs incubated at 40° and 41° C. noted blastoderms without embryos, defective heads, defective optic vesicles, spina bifida, failure of the somites to form. Kaestner ('95) performed an extensive series of experiments in which eggs after being incubated at normal temperature for various lengths of time were exposed to low temperatures for various periods, and then returned to normal incubation temperature. He found that when such developing eggs are kept too long at the lowered temperature, the time varying with the age of the embryo when exposed, abnormalities were obtained. They occurred most frequently when the temperature was lowered during the first two days of incubation. Disturbances of the head, heart, and brain were noted, and inhibition of such processes as the closure of the medullary folds, elevation of the head fold of the amnion, and union of the heart anlagen. Alsop ('19) incubated eggs at subnormal and supernormal temperatures and examined the embryos after one to three days incubation. At subnormal temperatures (94° to 102°), she noted failure of the neural folds to close, thickenings at the primitive knot, abnormal neural tubes often blocked by thickenings particularly in the lumbar region, curvature of the primitive streak. It seems probable to me from inspection of Alsop's figures that the thickenings noted are due to inhibition of the growing point of the primitive streak. At supernormal temperatures (104° to 108° C.) abnormalities of the brain, particularly of the optic vesicles and midbrain, were most frequent. Alsop's figures, however, also show inhibition in the primitive streak. In some cases extra somites appeared. Riddle ('23) exposed dove and pigeon embryos to subnormal temperatures for various periods during the first days of development but noted only a small percentage of abnormalities. I may here report a number of abnormal chicks obtained from eggs unintentionally left in the incubator room for several days before being placed in the incubator. The temperature of this room is about 30° C., a temperature at which, as is well known, the hen's egg will develop slowly for some time. These eggs were incubated about thirty hours: all of the chicks alive at the end of this period, about one dozen, were abnormal. The growing region of the primitive streak was in all cases inhibited to a greater or less extent. In most of the embryos it formed one or more irregular projecting masses of cells. As a result of this inhibition of the primitive streak, the posterior half of these embryos is too short and with too few somites as compared with the anterior half. One of these embryos is illustrated in Fig. 48. The anterior half of this embryo is normal (except the heart) but posteriorly the primitive streak terminates in a large projecting mass. On the right side the number of somites is about normal for the state of development of the anterior end but on the left side the number is considerably reduced. The posterior end of the neural tube is inhibited: the tube is widely open and the neural folds poorly developed especially on the right side. As already stated, eleven other embryos with similar conditions of the posterior end were obtained. Of these twelve embryos the anterior end appears entirely normal except in two cases where the head is exceedingly abnormal represented only by a crumpled mass at the level of the heart, the heart being then located anterior to this mass. This condition was called omphalocephaly by Dareste. One of the two embryos of this type obtained is illustrated in Fig. 49. The posterior end is nearly normal but the greater part of the head is invaginated into a mass containing crumpled fragments of neural tube. This mass is situated to the left of the embryo. The heart is at the anterior end. In the majority of these embryos the heart is abnormal.

The most complete description of abnormalities in the chick is that of Dareste in his well-known book. As Dareste found that all experimental procedures induce practically the same types of anomalies, he does not state in his description how the different abnormalities were produced. He lists the following as the chief abnormalities observed by him in the chick embryo. (1) Duality and other anomalies of the heart. Dareste correctly ascribed the double heart to an inhibition of the union of the two heart tubes. (2) Blastoderms without embryos. In these cases the blastoderm consists only of ectoderm and entoderm, mesoderm being absent. (3) Anomalies of the area vasculosa, absence of blood islands and blood vessels. (4) Anomalies of the amnion and the allantois, due to inhibition at the time when these membranes are forming. Dareste thought many other abnormalities were caused by the suppression of the amnion, but he was probably incorrect in this opinion. (5) Asymmetries of various kinds, as of the eyes and two sides of the head. (6) Spina bifida, correctly ascribed by Dareste to inhibition of the closure of the neural folds. (7) Omphalocephaly, a condition in which, due to suppression of the development of the head, the head is reduced to a crumpled mass bent on the yolk (hence the name omphalocephaly) and the heart is situated anterior to the head. A typical case of omphalocephaly is illustrated in my Fig. 49. Dareste notes that omphalocephaly is nearly always accompanied by grave defects of the brain, eyes, and olfactory sacs. (8) Various modifications of the brain and neural tube. (9) Modifications of the eyes. (10) Celosomia or protrusion of the viscera ventrally, due to failure of the lateral limiting sulci to close below. (11) Failure of development of the limb buds and other abnormalities of the limbs.

From this review of literature it seems evident that the described abnormalities in the chick correspond with the expectations from the susceptibility data in-so-far as the latter are available. It has been shown in this paper that the parts of the embryo most susceptible to toxic agents and hence expected to be most amenable to modification are the anterior and posterior ends of the embryonic axis, the special sense organs, particularly the eyes, unions, crests of folds, bends, turns, etc., and the limb buds. It will be seen from the long list of abnormalities just reviewed that these parts are indeed those which most frequently exhibit abnormal development after subjection to a variety of experimental conditions. The susceptibility of certain other parts which seem frequently to exhibit abnormal development, as for instance the area vasculosa, was not determined in my experiments and hence it cannot be stated definitely that the same explanation applies to such anomalies. However, it seems very probable that such is the case. The heart is treated in a separate paper which follows this one.

Finally it should be pointed out that both of two paired structures or the two halves of a median structure are not necessarily equally susceptible; consequently asymmetrical abnormalities are possible since, for instance, one eye may be slightly more susceptible than the other.

IV. Summary.

1. Differences in the time of death of different regions of chick embryos dying in toxic solutions are described. Chick embryos of various stages from the primitive streak through the third day of development were studied. In primitive streak and head process stages, there is a simple death gradient from the anterior to the posterior end of the embryonic axis. From the medullary plate stage on there are two regions of high susceptibility—the anterior end of the axis and the growing point of the primitive streak; from these two regions death progresses towards the middle of the embryo. Other structures showing high susceptibility at certain times are: the special sense organs, the limb buds, and all places where unions, folds, bendings, etc., are about to occur. A more detailed summary is given on p. 11.

2. Application of the results to normal development leads to the following suggestions, susceptibility or time of death being regarded as roughly proportional to metabolic rate:

a. There is a gradation in rate of metabolic activity along the primitive streak in an antero-posterior direction. This probably determines the cephalo-caudal progress of development.

b. The head process is probably merely a further development of the anterior end of the primitive streak.

c. The primitive streak (excluding the head process) forms all of the embryo from the first somite posteriorly.

d. Such developmental processes as the union of paired primordia, formation of bends and flexures, occurrence and closure of folds, etc., result primarily from cell activity and not from mechanical causes.

e. Intense metabolism is a necessary condition for active development.

f. The chordate embryo in general develops through the formation of two centers of activity, one of which is situated at the anterior end of the embryonic axis and develops into the head, and the other of which, more posteriorly located, is a growing point which progressively shifts posteriorly, depositing the trunk of the embryo anterior to it. This posterior center of activity is the dorsal lip of the blastopore in amphibian embryos, that portion of the teleost embryo which is situated in the germ ring, and the differentiating region of the primitive streak in the chick.

g. Whether or not the chordate embryo develops with a primitive streak appears to depend on the relative time of appearance of the posterior center of activity with respect to the time of gastrulation. If the posterior center is established at the time of gastrulation, development occurs without the formation of a primitive streak; if later, a primitive streak arises.

3. A review of the literature on induced teratological development in the chick is given and an explanation of such development is suggested on the basis of the present results. It is pointed out that those portions of the embryo shown in this paper to be most susceptible to lethal solutions also most frequently exhibit abnormal development under the action of agents of a general depressing nature. Such agents or conditions in proper concentration and duration affect the course of development in rough proportion to metabolic rate, those parts of the embryo requiring the highest metabolic rate for their normal development or having the highest rate at the time of application of the condition being more affected than parts developing at lower metabolic levels.

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PLATE I.

FIGS. 1 TO 3. Three stages in the disintegration of a chick embryo in the primitive streak stage showing course of disintegration from anterior to posterior end of the streak.

FIGS. 4 TO 6. Chick embryo in the head process stage, Fig. 4, disintegration of the head process, Fig. 5, and course of disintegration posteriorly along the streak, Fig. 6.

FIGS. 7 TO 10. Medullary plate stage. Disintegration begins in the primitive knot, Fig. 7, and progresses posteriorly along the streak, Figs. 8 and 9. It then attacks the anterior end of the medullary plate, Fig. 9, and proceeds backward along the plate, Fig. 10.

FIGS. 11 TO 13. Early neural fold stage. Disintegration similar to the preceding.

FIGS. 14 TO 17. Stage of the first somite. Disintegration begins in the anterior end of the primitive streak, Fig. 15, and proceeds in both directions from this region. It then attacks the anterior end of the neural folds, Fig. 16 and progresses posteriorly meeting the other disintegration, Fig. 17.

FIGS. 18 TO 20. Three somite embryo. Disintegration begins in the differentiating anterior end of the primitive streak and in the middle of the neural folds. From both places it progresses forward and backward. The high susceptibility of the middle of the neural folds foreshadows the approaching fusion of these folds.

FIGS. 21 TO 25. Embryo of five somites, showing the double gradient, from the primitive streak forward and from the anterior end of the neural axis backward.

FIGS. 26 AND 27. Embryo of five somites in which the place of fusion of the neural folds is evidenced by heightened susceptibility.

PLATE I.

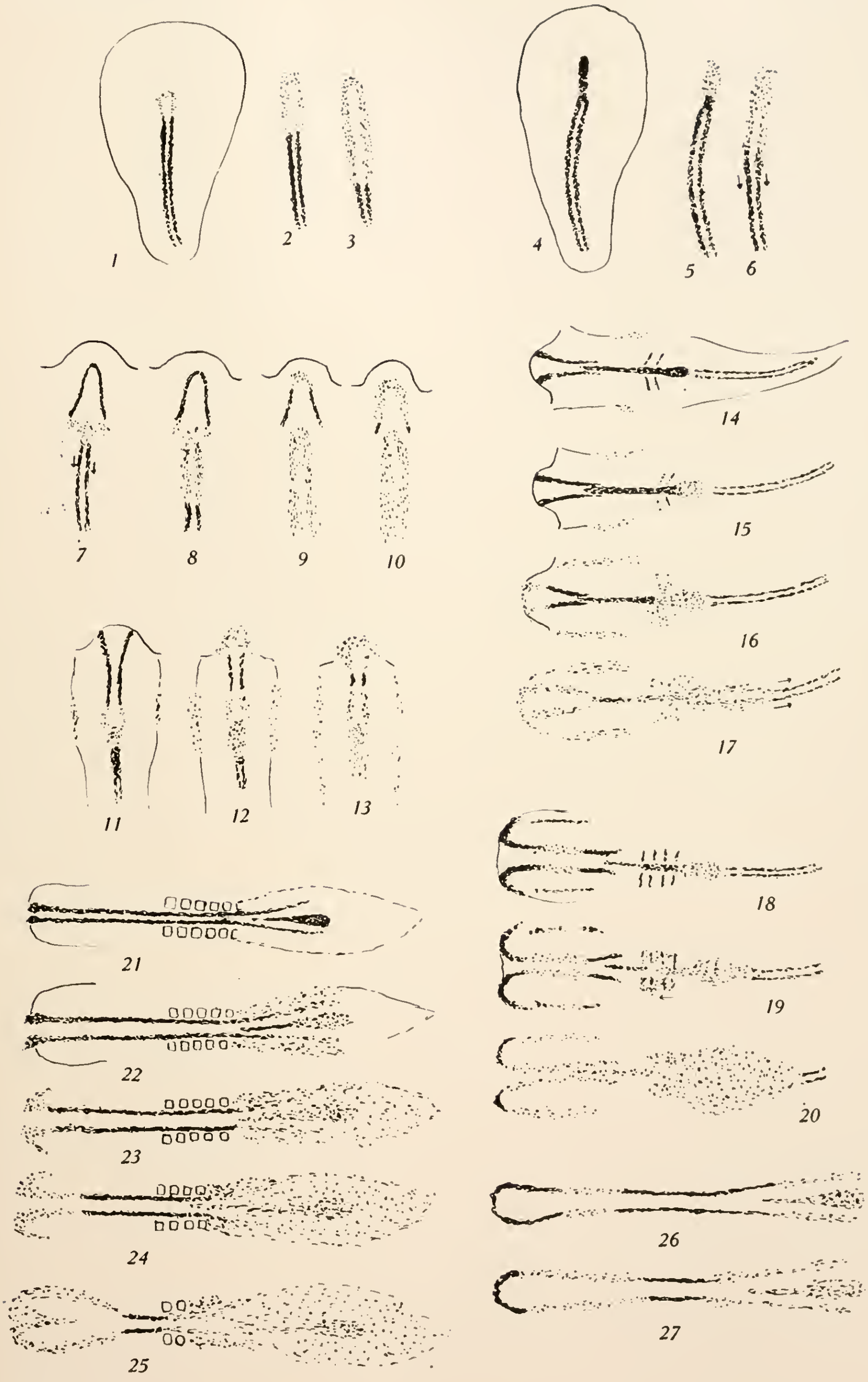
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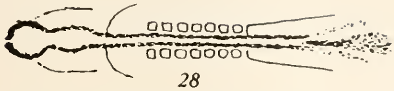
PLATE II.

FIGS. 28 TO 30. Embryo of seven somites, showing the usual double gradient of disintegration.

FIGS. 31 TO 35. Embryo of ten somites, showing the double gradient of disintegration and increasing susceptibility of the optic evaginations.

FIGS. 36 TO 39. Embryo of 12 to 13 somites showing general double gradient and area of increased susceptibility in the anterior end of the hindbrain foreshadowing the turning of the head.

FIGS. 40 TO 44. Fifteen somite embryo, with general double gradient; increased susceptibility of the optic stalks; increased susceptibility in the hind brain, more posteriorly located than in preceding figures, correlated with the turning of the head.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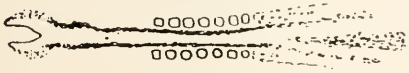
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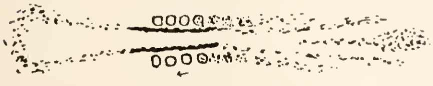
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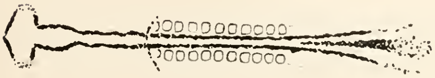
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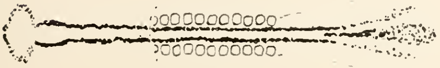
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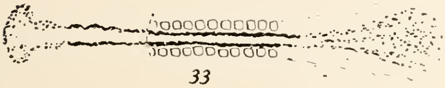
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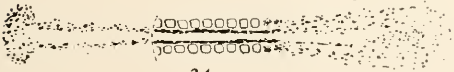
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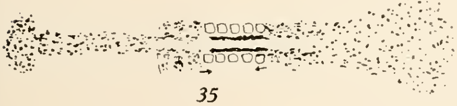
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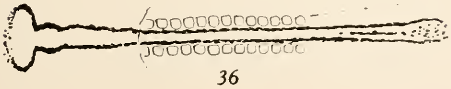
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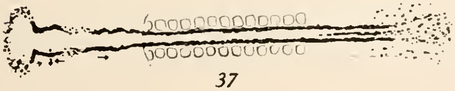
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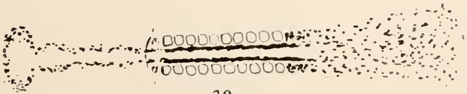
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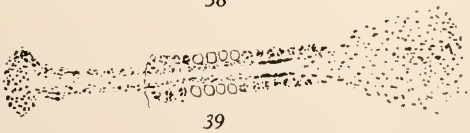
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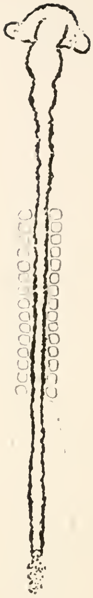
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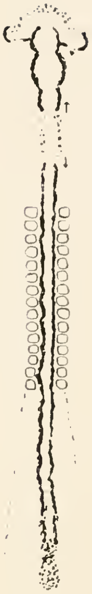
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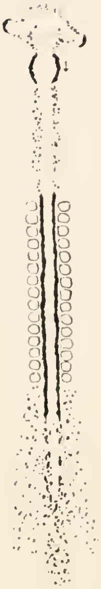
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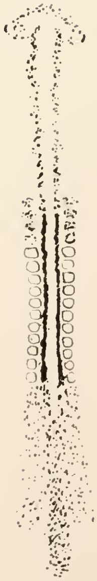
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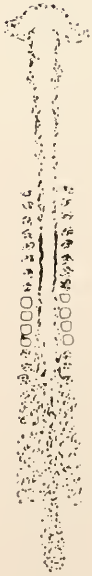
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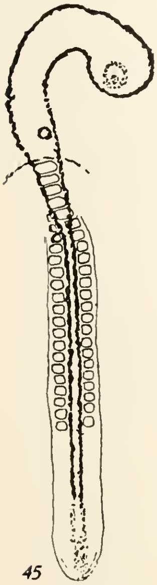
Plate III.

Figs. 45 to 49. Embryo of about two days incubation, with general double gradient, showing also high susceptibility of the eye and ear and of the region involved in the turning of the embryo.

Fig. 48. Abnormal chick resulting from retarded incubation. The anterior part is normal (except the heart), but the posterior end is retarded as shown by decreased number of somites on the left side, poor development of the neural folds, and large mass of cells terminating the embryo, due to inhibition of the growing region of the primitive streak.

Fig. 49. Typical case of "omphalocephalic" abnormality, due to delayed incubation. Posterior part nearly normal, but head reduced to a crumpled mass with heart anterior to the head.



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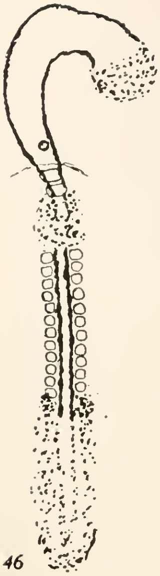

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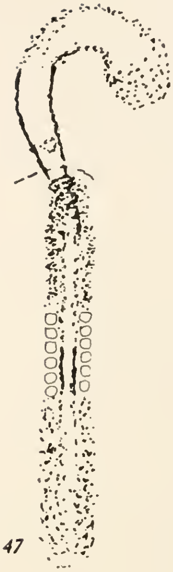

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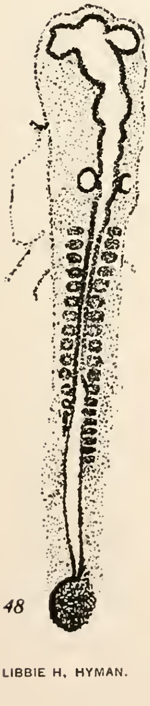

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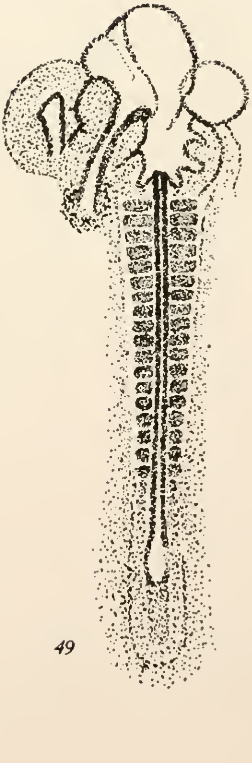

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LIBBIE H. HYMAN.

THE METABOLIC GRADIENTS OF VERTEBRATE EMBRYOS. IV. THE HEART.

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The study of the disintegration gradients of the heart was part of the work on the gradients of the chick embryo which has been presented in the paper which precedes this one in the same number of this journal. The investigation deals with the heart of the chick embryo only.

The methods employed were the same as recounted in the preceding paper of the series. The chick embryos were mounted ventral side up and the blastoderm which passes beneath the heart generally removed with a needle after the disintegrating agent had been applied and had begun to take effect. Unless this blastoderm is removed the heart cannot be seen very distinctly. The agents used were the same as in the preceding study, namely, potassium cyanide, ammonium hydroxide, and sodium hydroxide, all made up in rather strong concentration in isotonic sodium chloride solution.

It has proved necessary to spend a great deal of time and material in studying the death gradients of the chick heart. The reason for this is that the heart commonly dies without showing any very clear or obvious death changes. This seems to be due to some peculiarity of texture or consistency in the heart even in very early stages. Since the observer can naturally determine the time of death of a structure only when death changes of a visible nature occur, susceptibility conditions in the heart have been difficult to study. The clearest death changes occur in sodium hydroxide solutions but the same susceptibility differences have also been noted when ammonia or potassium cyanide solutions are employed.

Immediately after the establishment of the medullary plate the head of the chick embryo is delimitated from the blastoderm by the formation of the head fold. This fold composed of entoderm and ectoderm is semicircular in outline, its concavity directed posteriorly. "The head fold thus produces an internal bay in the entoderm, the beginning of the fore-gut" (Lillie, "Development of the Chick," 2d edition, p. 91). The posteriorly advancing crest of the head fold thus marks the posterior limit of the fore-gut and constitutes the floor of the anterior intestinal portal.

When embryos of two or three to five or six somites are studied in disintegrating solutions, ventral side up, it is seen that the central part of the semicircular crest of the head fold is the most susceptible and that susceptibility diminishes from the center posteriorly along the margins of the head fold. This is shown in my Figs. 1 to 3.

At about the stage of four somites the mesoderm begins to invade the head fold as the so-called amnio-cardiac vesicles. These two cavities push in towards the median line. In the splanchnic wall of each amnio-cardiac vesicle where it lies adjacent to the entoderm of the advancing crest of the head fold, a mesodermal tube appears. These two tubes are as is well known the primordia of the heart. Upon studying embryos at this time, about five somites, the disintegration of this region is the same as earlier. The central part of the crest of the head fold is the most susceptible and the susceptibility diminishes along the posteriorly extending margins of the fold which now contain the heart tubes. The death of this region of a five to six somite embryo is shown in my Figs. 1 to 3. The metabolic conditions of the heart-forming region at this time are thus in harmony with the fact that the two heart primordia fuse from before backward, a high metabolic rate being essential for the process of fusion, as seen in the preceding paper.

By about the stage of seven somites the two heart primordia have fused, so that a small heart is present. But the crest of the head fold, now perhaps more correctly designated the floor of the anterior intestinal portal, still retains its high metabolic rate. Consequently after the heart tubes have united the point of high susceptibility and metabolism is still the middle of the crescentic fold which marks the posterior extent of the fore-gut. This region, however, *formerly* at the *anterior* end of the heart tubes, is *now* at the *posterior* or *sinus* end of the heart. Consequently the death gradient in the heart now extends from the sinus towards the arterial end of the heart. In most of these early stages of the heart there is also still persistent more or less of a reverse gradient from the arterial end of the heart posteriorly. The extent of this differs in different hearts. In some it is very pronounced, in others scarcely evident. It seems probable that there is some growth in length at the arterial end of the heart.

The disintegration of a heart of a seven somite chick is shown in Figs. 4 to 8. Disintegration begins in the center of the posterior wall of the sinus, extends backward along the vitelline veins, forward along the heart towards the arterial end. At the arterial end there is a slight reverse gradient as shown in Figs. 6 to 8. Of four hearts of this age examined three were like the one illustrated while in the fourth the reverse gradient from the arterial end towards the sinus end was considerably more extensive.

At eight somite conditions in the heart are about as at seven somites, the reverse gradient being generally a little more pronounced. The typical course of disintegration at eight somites is illustrated in Figs. 9 to 11. Disintegration begins in the posterior wall of the sinus and progresses anteriorly along the heart. It then is initiated at the arterial end and proceeds towards the sinus end, meeting the other disintegration at about the middle of the heart. Of nine hearts of this stage examined, the reverse gradient was slight in three, extended about half way along the heart in four cases, and was entirely absent in one case. In one case there was hardly any difference in time of disintegration along the heart tube.

The heart of the nine somite stage is similar to the preceding but differs in one important particular. The disintegration commonly proceeds *faster along the right side* of the heart than the left. This is illustrated in the figures, numbers 12 to 16. Of nine hearts examined the right side was more susceptible than the left in five cases, in three cases the two sides were about equally susceptible, and in one case, the left side was more susceptible. In eight of them a reverse gradient from the arterial end towards the middle of the heart was present to a

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greater or less extent. The greater susceptibility of the right than of the left side of the heart may already be evidenced at the eight somite stage. Its interpretation is considered immediately.

Two points may be emphasized here. First, in these early stages before the heart has begun to beat, there is already present a gradation in susceptibility, *i.e.*, in metabolic rate, from the sinus towards the arterial end of the heart. This gradient appears to originate from the backward growth of the anterior intestinal portal, which, at the time of the formation of the heart, takes place by the fusion from before backward of the splanchnopleuric margins of the head fold. Thus the direction in which the heart beat will pass along the heart seems to be determined by the manner of development of the fore-gut of the embryo. The second point concerns the greater susceptibility of the right side of the heart. This condition undoubtedly foreshadows the bending of the heart to the right which occurs subsequently. As emphasized in the preceding paper of this series all such bendings are preceded by a higher metabolic rate on the side which is to become the convex surface of the bend.

At the stage of ten somites the greater susceptibility of the right side is generally very pronounced. The disintegration of a heart of a ten somite chick is shown in Figs. 17 to 21. Disintegration proceeds from the sinus along the right side, later on the left side. There is generally still present a slight reverse gradient at the arterial end. Thirteen hearts of this stage were examined, of which eleven exhibited this reverse gradient, eight to the extent shown in the figures, three to a greater extent.

At about the stage of eleven somites the heart begins to bend to the right. This condition is marked by a high susceptibility of the middle of the right side. Disintegration begins as usual in the sinus end of the heart but soon attacks the middle of the right side; from this point it then extends anteriorly and posteriorly along the right side, and laterally towards the left side. The disintegration of a heart of eleven somites is shown in Figs. 22 to 26. From this time on the reverse gradient at the arterial end is either absent or very slight in extent.

Hearts of twelve to fifteen somites are generally similar as regards their death gradients to the eleven somite heart, Figs. 22 to 26. In all a total of sixteen hearts of ages from eleven through fifteen somites were studied. In all of these the right side was more susceptible than the left, usually markedly so. In twelve cases, the gradient on the right side was of the type shown in Figs. 22 to 26, that is, disintegration extended from the middle of the right side anteriorly and posteriorly. In the other four the course of disintegration was similar to that shown in Figs. 17 to 21.

After the stage of fourteen somites the high susceptibility of the middle of the right side of the heart gradually lessens and has completely disappeared by sixteen somites, sometimes earlier. In hearts of embryos of sixteen to over twenty somites there is always present a simple gradient from the sinus to the arterial end of the heart. At this time of course the heart is beating regularly. It is interesting to note that the disintegration of the sinus always begins exactly at the places where the beat is initiated. The disintegration of a heart of a sixteen somite chick is shown in Figs. 27 to 31. The place of initiation of the beat is indicated in Fig. 27 by arrows. Ten hearts of chicks of sixteen to eighteen somites were observed and the course of disintegration was the same in all of them.

Conditions also continue the same from these stages through about the second day of development. Figs. 32 to 35 show the disintegration of a heart of a 20 somite chick. As before the sinus begins to disintegrate at the place where the beat originates and from there disintegration proceeds along the heart tube towards the arterial end where there is a slight reverse gradient. From the sinus death progresses caudally along the vitelline veins. Conditions remain the same up to about 25 somites, or the end of the second day of development.

From the second to the third day there is noticeable a greatly increased susceptibility in the ventricular region. The auricular evaginations also exhibit heightened susceptibility. Figs. 36 to 39 illustrate the disintegration of a heart of a thirty somite chick, about sixty hours of incubation. Disintegration begins at the tip of the ventricular bend and from there proceeds anteriorly along the bulbo-conus arteriosus and caudally towards the auricular region. Meantime the auricular evaginations are disintegrating. The sinus region at this stage cannot be seen very well as it is buried in the body. Stages beyond the third day have not been studied.

It thus appears that there is in the heart before it has begun to beat a gradient in activity from the sinus towards the arterial end and that this gradient is the cause of the direction and sequence of the heart beat. This gradient appears to be established simply by the mode of formation of the heart by the backward growth of the anterior intestinal portal, just as a similar postero-anterior gradient is established at the rear end of the chick embryo by the backward growth of the growing point of the primitive streak, as set forth in the preceding paper. In the latter case, the gradient does not persist and hence this embryonic postero-anterior gradient in the axis leaves only morphological signs behind it, namely, the segmentation of the vertebrate body. There may be some functional paths of the postero-anterior type also in the nervous system as permanent records of the embryonic physiological history. But in the heart the postero-anterior, *i.e.*, the sin-arterial, gradient is permanently retained. Why? We cannot answer this question very well except to suggest that the isolation of the heart from the dominance of other structures permits it to retain its high embryonic rate of metabolism.

In the history of physiology, much effort, argument, and paper have been expended on the question of the "cause" of the heart beat, whether neurogenic or myogenic or due on further analysis to the chemical conditions within the heart. This question like many other biological questions has never received any adequate or satisfactory answer; and the reason for this is, as in many other cases, that the question is a false one, it is wrongly put. It seems to me to be really not of the slightest significance whether the heart beat be neurogenic or myogenic; the real question is: what property of any tissue makes that tissue automatic? The crux of the matter would seem to lie in the nature of stimulation. It is evident that some organs function without extrinsic stimulation, while others do not.

Elsewhere I have made a suggestion as to the essential nature of the stimulated condition (Hyman, '18). I have pointed out that the chief difference between a stimulated and unstimulated organ is apparently one of rate of activity. A muscle or gland in a stimulated state does not appear to exhibit phenomena different from those characteristic of it in the unstimulated condition; it simply carries on its particular processes at a faster rate. If this be true then the essential feature of stimulation is an increase in the rate of processes in the organ stimulated. Granting this premise it follows that any organ whose rate of activity is already sufficiently high will of necessity be automatic. From this point of view the cause of the heart beat may be expressed simply as follows: certain or all of the heart tissue has so high a rate of activity that it functions in the absence of extrinsic stimulation. Now it is not of any consequence whether the tissue of the heart which possesses this high rate of activity is muscle or nerve. The state of affairs in this regard differs in different hearts. It is highly probable that in all hearts when they begin to beat in the embryo the automaticity resides in the muscle cells. But the muscle cells may lose their high metabolic rate with age and in that event the aid of the nervous system must be partially or wholly evoked to keep the apparatus going. The nervous system appears to be characterized by a high metabolic rate and is thus able to control absolutely or alter the rate of activities of other organs. In the process of ontogeny it comes to exercise more or less complete control over the heart, the degree varying in different animals.

It remains to consider briefly the teratology of the heart. Dareste in his book ('91) discusses only one type of anomolous heart in the chick, namely, the duplicature of this organ. This condition as he correctly points out results from a failure of the two heart primordia to unite. This union begins at the anterior end of the two heart tubes, a region of high susceptibility as shown in this paper. Depressing conditions would undoubtedly inhibit this union in whole or part resulting in the occurrence of partially or completely doubled hearts.

On the basis of the susceptibility data presented in this paper one might also expect certain other abnormalities of the heart. Thus I have shown that at an early stage of the heart the right side of the heart is more susceptible than the left. This greater activity of the right side results in the bending of the heart to the right. Depressing conditions would tend to prevent this bending, so that the heart would remain as a straight tube. I have also shown that in general the sinus end of the heart has the higher rate of activity and that this decreases towards the arterial end. Inhibiting factors might be expected to affect the sinus end of the heart more than the arterial end, so that hearts with small sinuses and enlarged arterial ends might be produced.

Both of these expectations have been realized in certain abnormal chicks produced during my study. These chicks have already been referred to in the preceding paper. They arose as a result of incubation for several days at 30° C., followed by incubation at 39° C. for 24 hours. That development had occurred at 30° is proved by the fact that all of these chicks showed a degree of development corresponding to 40 to 72 hours of incubation. All of this lot of chicks were abnormal and in many of them the heart was abnormal. As the other abnormalities were considered in the preceding paper, only the heart concerns us here. The abnormalities were of two kinds: (1) the persistence of the straight form of heart tube at a stage of development in which the heart would normally be considerably curved; and (2) a reduced sinus region with an abnormally enlarged arterial end. The first type of abnormality is illustrated in my Fig. 40. This embryo has thirteen somites but the heart lacks the normal curvature. The arterial end is also abnormally large. Fig. 41 is typical of the second kind of abnormality. In this embryo of seventeen somites the sin-auricular region is quite small as compared with the normal but the anterior part of the heart is considerably enlarged. This heart also does not bend to the right to the normal extent. It should be stated that all of these hearts were living and beating at the time when the embryos were fixed.

It thus appears that the teratological development of the heart corresponds with the expectations on the basis of the susceptibility results.

SUMMARY.

1. Before the formation of the heart the middle of the crest of the head fold is highly susceptible and the susceptibility decreases along the posteriorly directed margins of this fold which contain the primordia of the heart. This finding is in harmony with the fact that the two heart primordia fuse from in front backward.

2. After the heart is formed the anterior intestinal portal, now situated at the posterior end of the heart, retains its high susceptibility, and this fact appears to induce a corresponding direction of the gradient in the heart. In the heart at all stages from its first formation (about seven somites) to the latest stage studied (three days) there is a general susceptibility gradient from the sinus towards the arterial end. This gradient appears to originate from the manner of formation and backward progression of the anterior intestinal portal. In early stages of the heart there is usually also present a reverse gradient at the arterial end, due probably to the persistence of conditions stated in 1.

3. There is thus in the heart before it has begun to beat a sin-arterial gradient in metabolic rate. This is probably the cause of the sequence of the heart beat.

4. In addition to the general sin-arterial gradient, secondary regions of heightened susceptibility appear at times. The most important of these is the increased susceptibility of the right side. This begins at about the nine somite stage and persists until about fifteen somites. It is correlated with the curvature of the heart to the right. Between the second and third day ventricles and auricular evaginations show temporary local increased susceptibility.

5. Described abnormalities of the heart can be correlated with these findings on differential susceptibility along the heart.

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PLATE I.

FIGS. 1 TO 3. Ventral view of anterior end of an embryo of five to six somites, showing the disintegration of the crest of the head fold.

FIGS. 4 TO 8. Five stages in the disintegration of a heart of a seven somite chick, showing the sin-arterial gradient with a slight reversal at the arterial end.

FIGS. 9 TO 11. Course of disintegration of an eight somite heart, similar to the preceding, but with a more extensive reversal at the arterial end.

FIGS. 12 TO 16. Disintegration of a nine somite heart, showing general sin-arterial gradient and greater susceptibility of the right side as compared with the left.

FIGS. 17 TO 21. Disintegration of a ten somite heart, similar to the preceding, the greater susceptibility of the right side very marked.

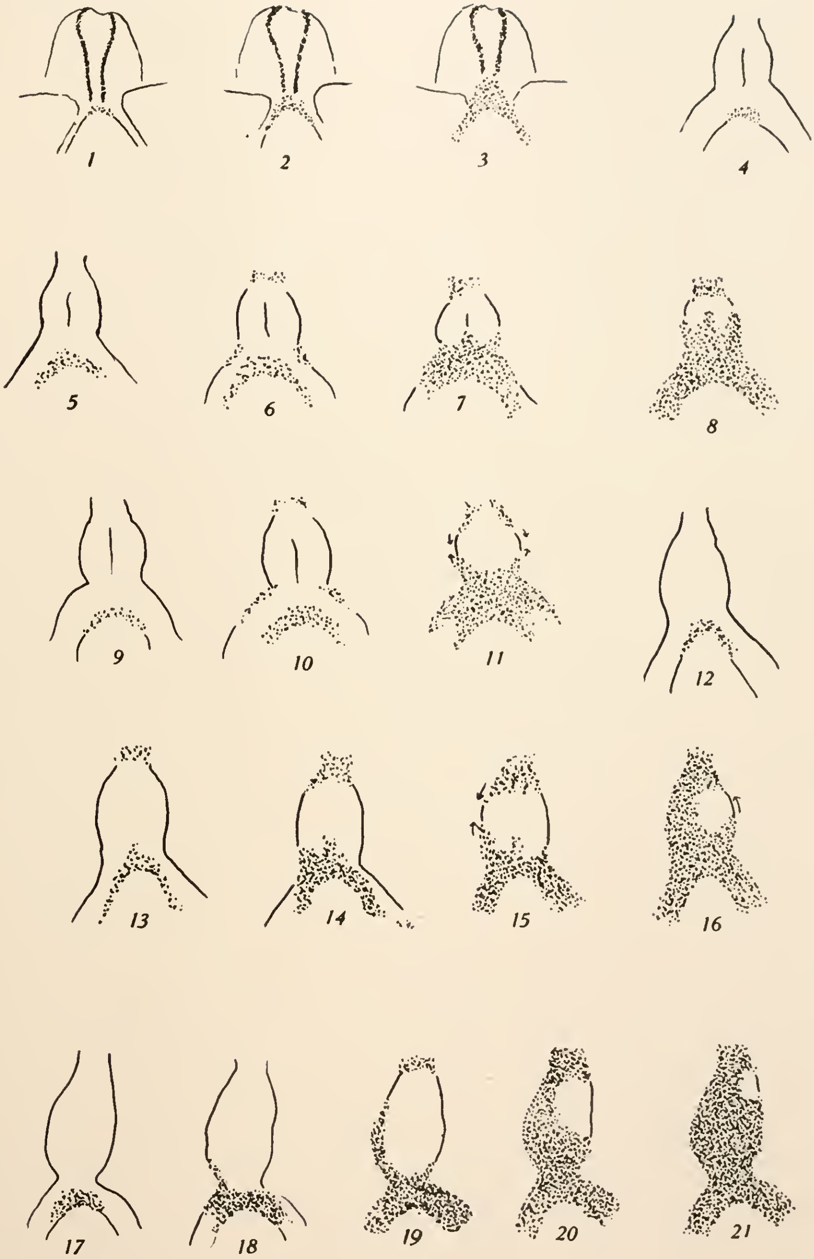
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Plate II.

Figs. 22 to 26. Disintegration of a heart of eleven to twelve somites showing increased susceptibility of the middle of the right side.

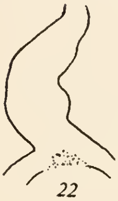
Figs. 27 to 31. Heart of a sixteen somite chick, showing restoration of simple direct gradient from the sinus to the arterial end. Disintegration in the sinus begins at the exact places of initiation of the heart beat, indicated in Fig. 27 by arrows.

Figs. 32 to 35. Heart of twenty somites, similar to the preceding.

Figs. 36 to 39. Heart of thirty somites, showing heightened susceptibility of the ventricular bend and the auricular evaginations, indicating rapid development of these regions at this time.

Fig. 40. Ventral view of an abnormal heart of a chick embryo of thirteen somites, obtained by incubation at subnormal temperature, showing inhibition of the bend to the right, and abnormally enlarged arterial end. The nervous system is normal but the posterior end of this chick (not shown in the drawing) is inhibited as discussed in the preceding paper.

Fig. 41. Ventral view of another abnormal chick of the same lot as Fig. 40 showing reduced sinus region as compared with arterial region. This chick also has an inhibited posterior end, not shown.



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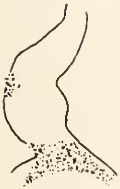

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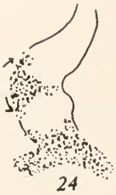

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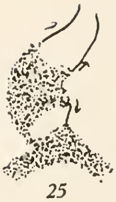

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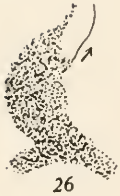

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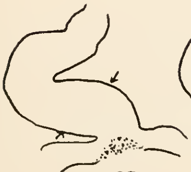

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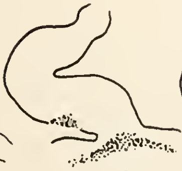

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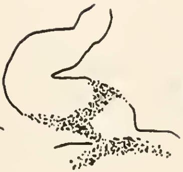

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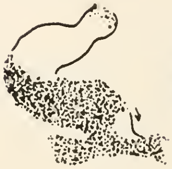

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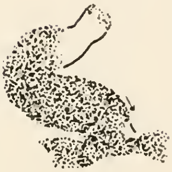

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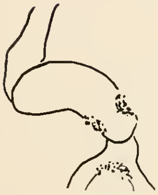

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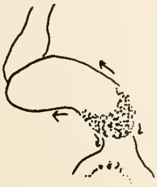

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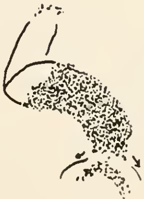

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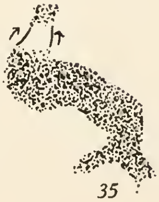

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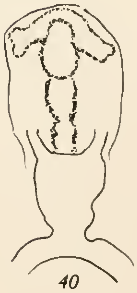

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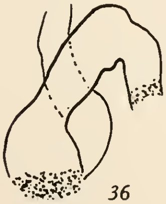

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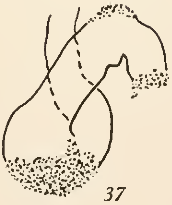

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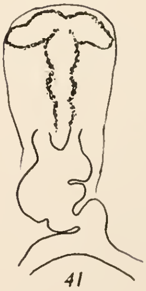

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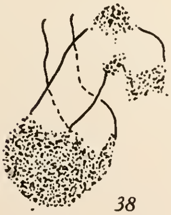

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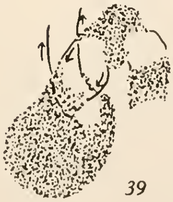

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LIBBIE H. HYMAN.

EXPERIMENTS IN REARING COLONIES OF BUMBLEBEES (BREMIDÆ) IN ARTIFICIAL NESTS.[1]

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Introduction.

In a detailed study of the life history of the bumblebees and their inquilines and parasites, it is obviously impossible to secure all the desired information from field observations alone. Therefore, various methods of procedure are necessary and some laboratory methods must be used. An account of the methods developed for the rearing of colonies of these social bees during five years of study may seem superfluous, but in view of previous publications, the ever-increasing interest in these insects, and their economic importance, such an account has its practical as well as academic value.

Until comparatively recent times practically all of our biological knowledge concerning the activities associated with nidification were the result of the chance discovery of nests, their hasty examination, and more rarely their observation for a limited period of time. To a large extent the classical observations of Réaumur (1742), Huber (1801), Lepeletier (1836), Putnam (1864) and many others were the result of this type of study. It is but natural, then, that the information so accumulated should relate for the most part to the size of the colonies, the inquilines and parasites found in the nest when opened, the arrangement of the comb, and various other readily apparent details of the nest economy. Such a method, however, can not be depended upon when a more detailed study of the life history of the individual bees and the development of the colony of one or more species is desired. Furthermore, the fact must not be overlooked that the vast majority of the colonies discovered in their natural environments are found at a time of the year when little can be learned about many of the aspects of their life history; the starting of the colony, for example.

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[1] Contribution from the Entomological Laboratories of the University of Illinois, No. 106. Extract number three from a thesis presented to the Faculty of the Graduate School of the University of Illinois in May, 1923, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

Hoffer (1882–1883) says on page eleven of the first part of his celebrated publication that he has never seen how the comb is started and adds that apparently neither has any other observer. In the appendix of the second part of this work, however, Hoffer tells how to his great joy he finally observed the start of the comb of *B. lapidarius*. In this latter instance the queen was under confinement and the information obtained by Hoffer concerning this phase of the development of the comb was not based on field observations. Westerlund (1898) has given us a short account of a nest in its incipient stages that he found in Finland and v. Buttel-Reepen (1903) gives a few notes on the subject furnished him by Herr Wegener. Since then Wagner (1907), Lindhard (1912), Sladen (1912), Armbruster (1914) and Plath (1923) have added to our knowledge concerning the start of the comb. Sladen and Plath give the best accounts of the start of the nest and comb thus far published. The correctness and completeness of the descriptions of these last mentioned writers are due to the fact that they did not rely upon field observations alone.

By calling attention to the disadvantages incurred by depending upon field nests and the limitations of such studies I do not mean to imply that the examination of such nests is not or has not been of importance. I merely wish to point out that by so doing one is relying to a great extent upon chance and therefore a means which does not guarantee results to one intent upon penetrating deeper into many of the details, characteristics, and mysteries of the life history of the bumblebees.

In a comparatively recent number of the BIOLOGICAL BULLETIN (1923), Mr. O. E. Plath has published an exceedingly interesting and valuable account of his "Breeding Experiments with confined *Bremus* (*Bombus*) Queens." In the introductory part of this paper Plath adequately reviews the experiments of Hoffer (1882), Lindhard (1912), Sladen (1912), and Frison (1918) of this nature, and accordingly these papers need not be reviewed again.

Construction of Artificial Nests and Technique.

My first serious experiments in confining the queens began in the spring of 1915 and were continued each spring of 1916, 1917, 1919, and 1920. As early as 1910 I tried to get queens to start nests in confinement, but the methods then employed were so poor that I will not review these futile attempts. It was not until 1917 that I succeeded in getting colonies by this method (1917). For confining the queens I used, in 1917, a small wooden box 5 inches by 7 inches by 2 inches. At one end of this box was a round opening which could be closed by the insertion of a cork. For the top I used small ruby or amber colored glass sections. During the intervals between examinations a dark cloth was wrapped about the box to exclude the light. In one corner of the box remote from the grass nest was placed a small tin, in which liquid food was supplied to the queen. In 1919 and 1920, I used boxes for confining the queens similar to those used in 1917.

Within these boxes I used various materials for making the conditions such that a queen would be induced to start a colony. Naturally, because the queens of bumblebees select the nests of field mice the first material used was the soft, day grass from such nests. Field mice always select a fine quality of grass for their nests and hence grass from such a source needs no further sorting or treatment. The nests, however, are not always easy to find and oftentimes I was hard pressed to get enough of the grass from this source for my experiments. Sladen experienced the same difficulty in getting suitable grass to use in domiciles for attracting bumblebee queens. I found that the best time and place to look for nests of this type was in early spring along a railroad right of way, after the ground had been burned over to destroy the coarser vegetation. The nests so located usually escaped destruction by the fire and were then easily perceived. The difficulty experienced at times in getting enough of these nests, led me to try to find a substitute for the finely sorted grass they contained. Cotton did not have enough "body" and the small cotton fibers became enmeshed continually with the pollen supplied the queen. The silk from milkweed pods likewise proved to be too light and fluffy, as was the case also with raw wool. In several instances I used in my boxes without alteration, except for the addition of a top covering, the nests of small birds such as those made by field sparrows.

Bumblebees always keep the brood and comb well covered, in order to exclude light, avoid temperature fluctuations, etc. When making daily observations on a colony I always found it necessary to remove the covering of the nest. When it was replaced again the workers or queen would immediately start to work and repair it. To save the bumblebees the daily repetition of repairing the top covering, I devised a square pad of four or five layers of cheese cloth, fastened these together and then placed this pad over the comb. This gave very good results. All I needed to do in order to see the contents of the nest was to lift the cheese-cloth pad, and then upon finishing my observations, replace the same. The bumblebees readily accepted this substitute for the covering of their own making and in so doing saved themselves from constantly repairing the top. As was the case with the grass top, the bumblebees plastered a thin coat or layer of wax and pollen composition on the side of the pad adjacent to the brood chamber. Later I went a step farther and coated one side of the cheese cloth pad with pure melted honeybee wax before placing it over the nest. This seemed to break the bumblebees of their habit or instinct of coating the pad with wax and pollen and hence resulted in a considerable saving to them of time and materials. The fact that the bumblebees accepted the artificial wax-lined cheese cloth pad without material change is a good indication that it fulfilled all the requirements of their instincts in this direction.

The artificial top proved to be such a decided success that I next tried to make the whole nest of cheese cloth coated on the inside with wax. I first tried lining the bottom of the box with cheese cloth, and then lining this with the melted wax. Then I placed over this a dome-shaped cover of wax-coated cheese cloth. Of course an entrance into this enclosed chamber was provided. Because of certain undesirable features I abandoned this method. Finally, I resorted to lining merely the bottom and sides of the back part of the box with four or five layers of cheese cloth and then coating this with melted wax. For the back and ceiling a single elongate pad of the right dimensions was made in the same manner as before, and then fastened to the back of the box so as to form a stationary back part and a movable covering. This could then be lifted up from the front in much the same way as one would lift the hinged lid of a box. This top pad was easily pinned to the floor in the fore part of the box, thus enclosing a neat light-proof, wax-lined and soft-walled chamber. An entrance to this enclosure was provided by cutting out a part of the top covering, or merely by pinning to the side of the box one corner of the free end of the top.

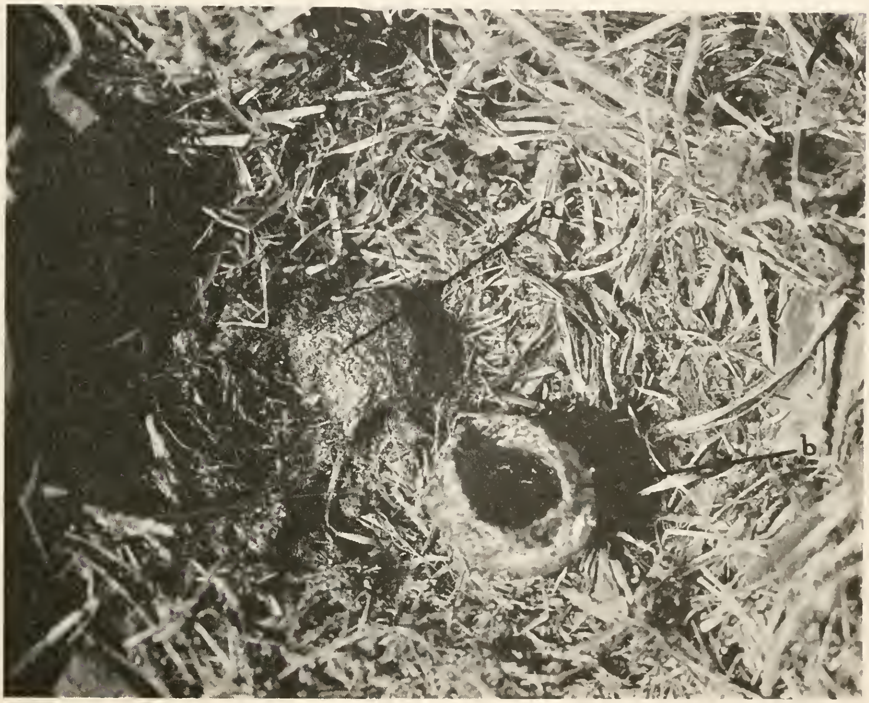

FIG. 1. Start of the comb of *Bremus impatiens* under controlled conditions, showing: *a*, the pollen lump containing the first egg cells and eggs; *b*, the artificial honey pot. May 2.

The adoption by the bumblebee queens of this last-described artificial nest relieved me of many of my earlier worries. I no longer needed to search about in spring for the nests of field mice, for this artificial nest seemed to fulfill in every way all the requirements of the nesting instincts which are so pronounced in bumblebee queens looking for a place to adopt as their home. Further, I could purchase very cheaply all the material that I needed for making these artificial nests. As a saver of time the artificial nest was a great improvement over anything I had ever used before. The nests could be made up at any time of the year, in any number and during odd moments. Formerly, I had to look for grass suitable for my purpose in spring, the very busiest time of the year for one attempting a biological study of bumblebees.

A slight variation of this artificial nest was often used. It consisted essentially of dividing the box into two sections, by means of a wooden partition. One of these sections was then made into an artificial nest as described above and a hole made in the partition so that the bumblebees could go from one section to the other. The remaining section was then used solely as a feeding chamber.

The liquid food which must be supplied to a queen bumblebee in confinement was kept in a small tin container. In the unpartitioned box this container was placed in one corner, and in the divided type in the section adjacent to the artificial nest. The liquid food supplied to the confined queens was simply pure, strained honeybee honey diluted with water. A mixture of sugar and water is eagerly consumed by hungry queens, but does not compare for this purpose with diluted honey, principally because it is not so concentrated. Adult bumblebees as well as the larvæ feed on pollen, but sometimes in confinement the queens can not be induced to eat the honeybee pollen. To defeat this indisposition on the part of the queens to feed on pollen, I occasionally added a small amount of rye flour to the diluted honey. In this way I hoped the queens would get some of the rye flour when lapping up the honey mixture. Pollen is rich in nitrogen and when eaten by the queens in spring probably contributes to the development of the eggs, causing the queen to become "broody" and finally to seek a home. Rye flour is also rich in nitrogen, and hence might aid in bringing about the same effect. The receptacles for the liquid food should permit as little exposure of the liquid as possible. Bumblebee queens frequently climb up the sides of the box and then, reaching the top and unable to go farther, fall down again. If by chance they get sticky because of falling into the tin cover containing the honey, there is less chance of the queen starting a colony. Bumblebee queens under natural conditions keep themselves scrupulously clean. Any substance such as honey which mats down their glossy pubescence and becomes a "catch-all" for particles of dirt readily tends to make the queens dissatisfied with their quarters and as a result no comb is started. I often partially covered with stiff paper the tin lid containing the honey, leaving but a little space for the insertion of the proboscis of a bee. Almost any contrivance of this nature would suffice. If one were to confine large numbers of queens it might be profitable to devise a neater and more efficient type of feeder.

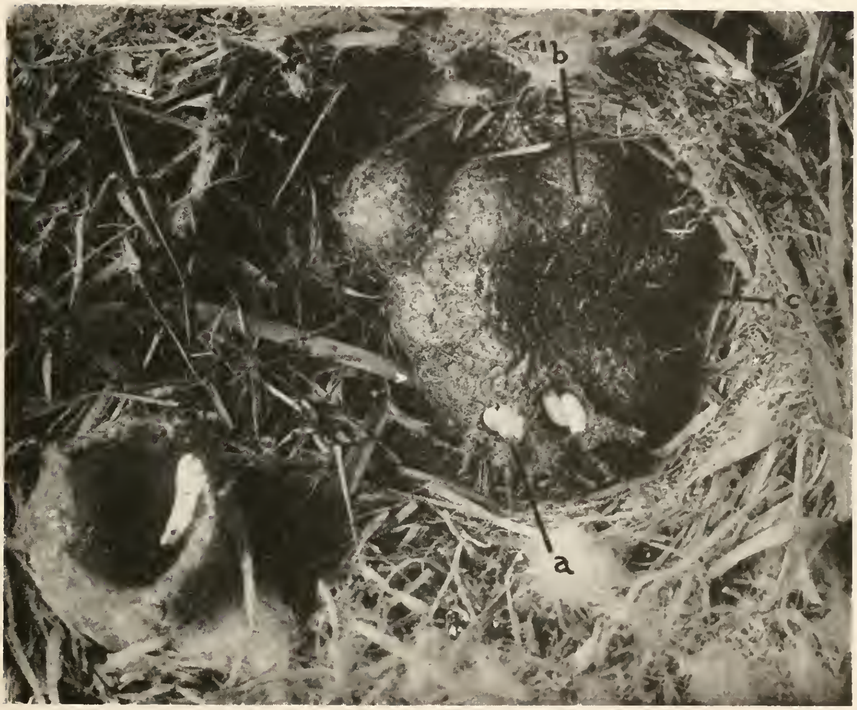

FIG. 2. Comb of *Bremus impatiens* of about two weeks development, showing: *a*, several eggs in the same cell; *b*, one of the first larval cells; *c*, groove caused by the queen brooding on the first larval cells. May 10.

Not too much of the diluted honey should be made up at one time as it rapidly sours. I found it convenient to make up this mixture by filling a four-ounce bottle about three fifths full of honey, adding enough distilled water to nearly fill the bottle and shaking it until the honey was thoroughly diluted. A cork stopper provided with a medicine dropper was useful for drawing the diluted honey from the bottle and refilling the tin containers in the nesting boxes.

Besides liquid food, pollen is essential to a brooding queen for her personal consumption and for the proper development of her off-spring. Honeybee pollen is apparently as wholesome for bumblebees as for honeybees. One spring I tried to secure fresh pollen by removing the pollen from the corbiculæ of worker honeybees which I caught in the field. This required too much time for the quantity secured; in fact, did not produce enough for my needs. Therefore, I relied mainly upon getting the pollen from the brood frames of the honeybee. The pollen should be carefully removed, so as to avoid being mixed with foreign substances, and kept in bottles until needed. If it dries out it can be moistened again with honey. I am much indebted to Dadant and Sons, of Hamilton, Illinois, for gratuitously furnishing me with pollen in the spring of 1920.

There is no actual difference between the pollen stored by the honeybee and that of the bumblebee unless it be in the nature of the substance or fluid used to moisten the pollen in order to make it adhere to the pollen plate. This is evident from the fact that much of the pollen gathered by both bumblebees and honeybees is from the same flowers.

Before using the pollen I mixed it with enough honey to give it the consistency and plasticity of pollen freshly scraped from the legs of a foraging bee. I then placed a lump of the pollen about the size of a cherry seed in the artificial nest. This corresponded to the pollen mass collected by a queen under natural conditions when about to lay her first eggs. Besides this, I usually placed a small amount of the pollen near the liquid food as an extra source of pollen supply. That placed in the nest was intended primarily for the queen to use in building her first egg cells, but the use of this also as food did not complicate matters.

Another feature of the artificial nest was the honey pot. This was made from pure melted beeswax and modeled to imitate in every respect the honey pot first constructed by a nesting queen. I fastened this to the bottom of the nest by means of melted wax, just about one half inch distant from the pollen lump which was to serve as a future egg receptacle. Diluted honey was used for filling the honey pot. Naturally, all the wax used by wild bees is produced by them, but to make conditions even more favorable I placed a few loose bits of pure beeswax near the artificial honey pot for the use of the queen if she so desired.

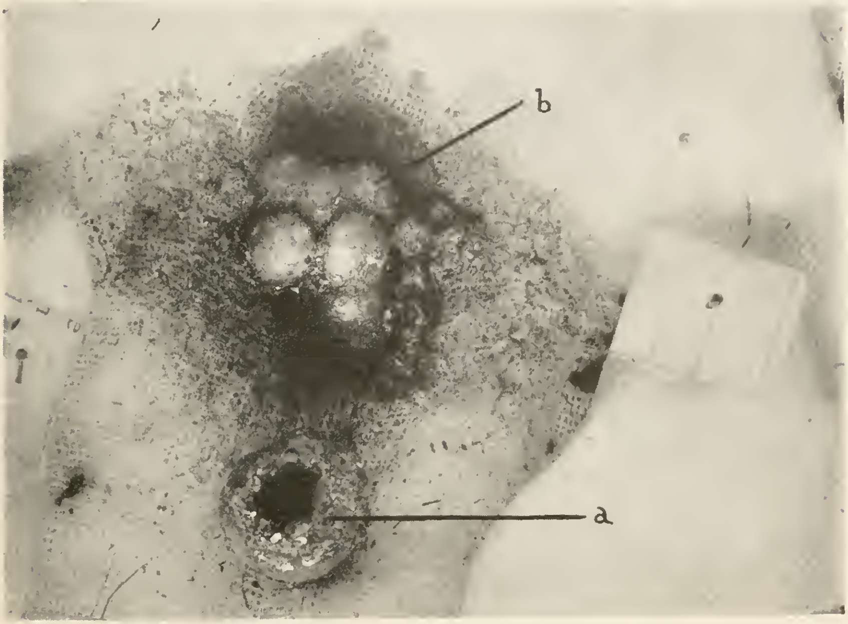

FIG. 3. Start of the comb of *Bremus vagans* in artificial nest, showing: *a*, artificial honey pot; *b*, first larval cells. June 21.

Considering the artificial nest as described above with all its parts, we find that it provides a queen with everything necessary for starting and eventually developing a colony. The wax-lined, cheese cloth chamber in the small wooden box in a general way corresponds to and possesses the same advantages as the nest of a field mouse. Under natural conditions after finding a suitable nesting situation the queens must gather pollen and nectar. The honey pot is constructed of pollen and wax and filled with honey, shortly after the pollen is gathered for the nucleus of the first brood mass. Occasionally I moistened a portion of the nest-lining with honey just to give it a bee-like odor. After I had placed an artificial honey pot, little bits of wax, and a small, compact mass of pollen in my artificial nest, there remained for the queen bent on laying eggs only the construction of the egg cells. Bumblebee queens under natural conditions avoid as much labor as possible. This is shown by the selection of nests of field mice, birds and other already

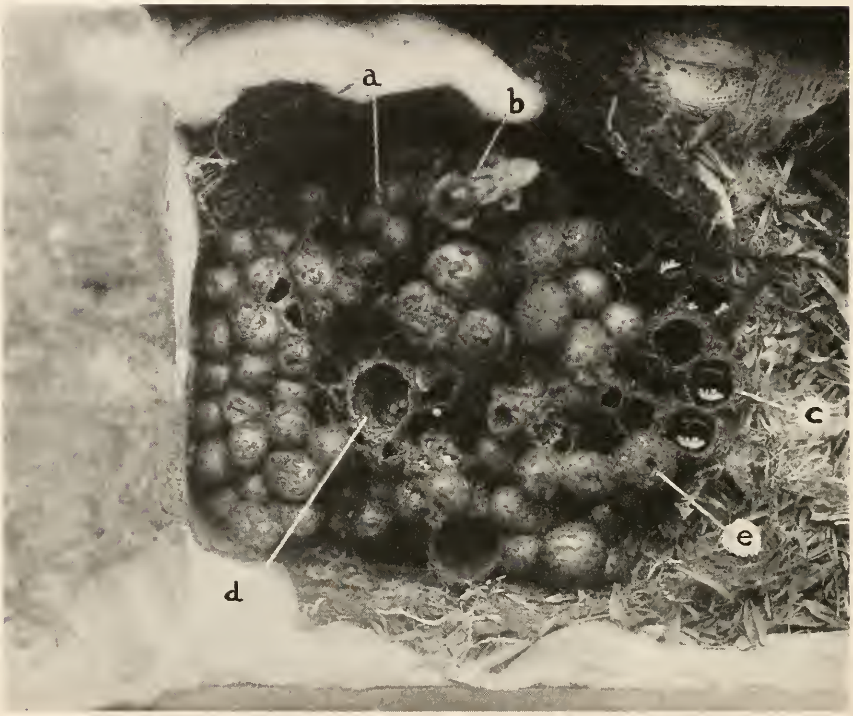

FIG. 4. Comb of *Bremus vagans*, showing: *a*, larval cells; *b*, old queen; *c*, wax-pollen pots used for the storage of honey; *d*, cocoon used for the storage of pollen; *e*, larval cell showing opening through which food is injected into the cell. September 16.

prepared quarters. Therefore, the elimination of the work of selecting a nest, the construction of a honey pot, and the collection of nectar and pollen should prove no obstacle to inducing the queens to start colonies in confinement. In fact, my experiments prove that such provisions are the secret of success in rearing bumblebee colonies in captivity. The method involved is simply a case of substituting artificial materials and objects for those naturally found or made by the queens. In my artificial nest as described above the queen finds herself in a position or environment which would be attained naturally only after days of patient labor. The instincts associated with the early start of a colony are so fixed that the queens often proceed with work in these artificial nests that is unnecessary. As an example of this I may state that bumblebee queens under such conditions often proceed to make changes in the artificial honey pot and in some instances build a new one.

The artificial nests should be prepared in sufficient numbers before the queens are on the wing in spring, except that the pollen lump should not be placed in the nest until the queens are introduced. I have always kept a close watch on the advance of the spring season and almost the first day of the appearance of the queens caught them for my experiments. In the vicinity of Urbana, Illinois, the queens of various species appear at different times and in order to get queens of all the species commonly found in this locality, it is necessary to prolong the collecting period over an extended time. The queens were captured in an ordinary insect net and then brought alive to the laboratory in mailing tubes. On arriving at the laboratory the queens were immediately removed and placed in large glass aquarium jars. Corrugated paper was placed on the bottom of these jars to afford the queens a suitable walking surface and the top of the jar was covered with several layers of cheese cloth to prevent their escape. Food was supplied them by scattering small bits of pollen about the bottom of their prison and by placing diluted honey in a tin-container fastened in the center of the corrugated bottom. In these jars the bees found plenty of food and ample opportunity for exercise. Besides keeping the queens at all times well fed, exercise seems to be essential.

Therefore, the larger the aquarium jars the better. If the queens are kept in too close quarters, even though provided with sufficient food, they soon become lethargic and die. Queens kept in the right type of aquarium jars and well fed soon become "broody." By "broody" I mean that the physiological processes going on within the bodies of the queens cause them to display characteristic nesting instincts. This is externally expressed by the production of wax, embracement of lumps of pollen in the jar, and excited buzzing and agitated movements when disturbed. Some species respond more quickly to this treatment than do others.

As soon as the queens became broody I put them in artificial nests. Usually two queens were placed in each nest, but I conducted also ten experiments in which only one queen was placed in each nest. Needless to say, when two queens were placed in the same nest, they were usually of the same species, but in some cases I purposely placed queens of different species in the same nest-box. Before introducing two queens into the same nest it is advisable to keep them in the same aquarium jar. In this way they appear to acquire the same characteristic odor and when placed in the nest the danger of the queens fighting is somewhat lessened. Where two queens were kept in the same box, in order to be able to distinguish them apart, I marked one of them by clipping a notch in her wings. In this way it was possible to keep an accurate account of the actions of each queen. Each artificial nest was given a number when one or two queens were introduced into it and observations made and notes taken as often thereafter as possible.

Results.

In Table I. is given the results that I obtained in getting queens to start colonies in captivity. In 1915 and 1916, the methods employed were very unsatisfactory and the fact that I did not succeed in starting a single colony is not surprising. Under "Remarks" in Table I. it is to be observed that in 1915 in two of the experiments the queens at one time gave some evidence of being interested in the nest. Though these experiments are classed as failures they, nevertheless, gave me many suggestions

TABLE I.

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. impatiens	1	1 queen	April 30, 1915	Queen died May 25, 1915	Manifested slight interest in the nest
B. auricomus	2	1 queen	April 30, 1915	Queen died June 23, 1915	No interest shown in the nest
B. americanorum	3	1 queen	April 30, 1915	Queen dead May 5, 1915	No interest shown in the nest
B. impatiens	4	1 queen 3 workers	May 25, 1915	Queen dead June 1, 1915	Interest taken in the nest at first, especially by workers
B. americanorum	5	1 queen	April 23, 1915	Queen dead April 30, 1915	No interest shown in the nest
B. impatiens	6	1 queen	April 23, 1915	Queen dead May 2, 1915	No interest shown in the nest
B. impatiens	7	1 queen	May 5, 1916	Queen dead May 7, 1916	No interest shown in the nest
B. separatus	8	1 queen	May 5, 1916	Queen dead May 27, 1916	No interest shown in the nest
B. bimaculatus	9	2 queens	April 17, 1917	Colony successfully started and reared	
B. bimaculatus	10	2 queens	April 17, 1917	One queen died May 1, 1917 One queen dead May 10, 1917	Interest manifested in the nest, but no eggs or cells. First queen killed by the other queen
B. bimaculatus	11	2 queens	May 5, 1917	One queen died May 11, 1917	No interest shown in the nest
B. bimaculatus	12	2 queens	May 13, 1917	One queen stung and partially paralyzed on May 16, 1917. Other queen released later	One queen took an interest in the nest about the time the first queen was stung
B. bimaculatus	13	1 queen	April 19, 1917	Placed with Exp. 14 on April 27, 1917	Interest taken in the nest as shown by making a "cozy" pocket in the nest, but then abandoned
B. bimaculatus	14	1 queen 1 queen	April 19, 1917 April 27, 1917	Colony successfully started and reared	
B. bimaculatus	15	2 queens	April 20, 1917	One queen died May 9, 1917 One queen released on May 15, 1917	No interest shown in the nest
B. bimaculatus	16	2 queens	April 20, 1917	One queen stung and killed on April 23, 1917. One queen released on May 5, 1917	One queen interested in nest on April 23, 1917, but lost interest after that date
B. separatus	17	1 queen 1 queen	April 20, 1917 April 24, 1917	One queen dead on May 13, 1917. Other queen placed in Exp. 18 on May 18, 1917	No interest shown in nest

TABLE I.—*Continued.*

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. separatus........	18	2 queens	May 18, 1917	One queen stung and paralyzed on one side on May 24, 1917. One queen released on June 26, 1917	No interest shown in nest
B. americanorum....	19	2 queens	May 15, 1917	One queen dead on May 23, 1917. One queen released on June 3, 1917	No interest shown in nest
B. americanorum....	20	2 queens	June 21, 1917	One queen dead on July 5, 1917. One queen released on July 8, 1917	No interest shown in nest
B. bimaculatus......	21	2 queens	April 17, 1917	One queen killed on June 11, 1917 after several workers had emerged. Colony successfully reared	
B. auricomus.......	22	2 queens	May 14, 1917	One queen removed after first workers had emerged. Colony successfully reared	
B. bimaculatus......	23	1 queen	April 10, 1919	One queen removed on May 30, 1919. Colony successfully reared	
		1 queen	April 14, 1919		
B. bimaculatus......	24	2 queens	April 15, 1919	One queen removed on May 12, 1919. Colony successfully reared	
B. bimaculatus......	25	1 queen	April 16, 1919	One queen stung and killed on April 30, 1919. One removed on May 14, 1919	No interest shown in the nest
		1 queen	April 17, 1919		
B. bimaculatus......	26	2 queens	April 21, 1919	Colony successfully started. Discontinued experiment	
B. impatiens........	27	1 queen	April 21, 1919	Colony successfully started and reared	
B. impatiens........	28	1 queen	April 21, 1919	*B. impatiens* dead on May 30, 1919	No interest shown in the nest
B. auricomus.......	29	2 queens	April 25, 1919	Colony successfully started and reared	
B. separatus........	30	2 queens	May 12, 1919	One queen removed on May 15, 1919. Colony successfully started and reared	
B. separatus........	31	1 queen	May 15, 1919	Queen removed from the nest on June 13, 1919	No interest shown in the nest
B. impatiens........	32	1 queen	May 16, 1919	Colony successfully started and reared	
B. auricomus.......	33	1 queen	May 24, 1919	Queen died on June 13, 1919	No interest shown in the nest
B. impatiens........	34	1 queen	May 25, 1919	Colony started by *B. impatiens* queen adopted and successfully reared by *B. auricomus* queen	
B. bimaculatus......		1 queen	June 8, 1919		
B. auricomus.......		1 queen	June 9, 1919		
B. fervidus.........	35	2 queens	May 29, 1919	One queen removed on June 1, 1919. Other queen laid eggs, but finally deserted the nest	

TABLE I.—*Continued.*

Species.	Experiment Number.	Number of Queens and Workers.	Date Experiment Started.	Results.	Remarks.
B. separatus	36	2 queens	May 29, 1919	One queen escaped on May 31, 1919. Another queen escaped on June 16, 1919. One queen laid eggs and partially reared larvæ, but finally deserted the nest	
		1 queen	June 1, 1919		
B. separatus	37	2 queens	May 31, 1919	One queen *B. separatus* killed on June 12, 1919. Queen of *B. vagans* died on July 6, 1919 due to nematode infection	No interest shown in nest by any of the queens
B. separatus		1 queen	June 11, 1919		
B. vagans		1 queen	July 5, 1919		
B. separatus	38	2 queens	May 31, 1919		No interest shown in the nest
B. vagans	39	2 queens	May 31, 1919	One queen removed on July 5, 1919. Colony successfully started and reared	
B. fervidus	40	2 queens	June 12, 1919	*B. fervidus* queens all died at various times in latter part of June and July	*B. fervidus* queens made egg-cells on two different occasions, but each time deserted the comb
B. fervidus		1 queen	June 15, 1919		
P. laboriosus		1 queen	July 6, 1919		
B. fervidus	41	2 queens	June 12, 1919	One queen dead on June 14, 1919. Nest successfully started but not reared	
B. bimaculatus	42	2 queens	May 3, 1920	One queen removed on May 16, 1920. Colony successfully started	
B. bimaculatus	43	2 queens	May 3, 1920	One queen removed on May 16, 1920. Colony successfully started	
B. ternarius	44	2 queens	May 8, 1920	Queens escaped from nest on June 13, 1920	No interest shown in the nest before queens escaped
B. terricola	45	2 queens	May 30, 1920	One queen killed on June 4, 1920. Remaining queen constructed egg-cells but died on June 6, 1920	
B. terricola	46	2 queens	May 13, 1920	One queen removed on May 15, 1920. Colony successfully started	
B. perplexus	47	1 queen	May 30, 1920	Colony successfully started	
B. ternarius	48	2 queens	June 3, 1920	One queen removed on June 8, 1920. Colony successfully started	
B. impatiens	49	1 queen	May 30, 1920	Colony successfully started	
B. perplexus	50	1 queen	May 16, 1920	Colony successfully established	
B. centralis	51	2 queens	June 6, 1920	Large egg-cells made, but no eggs laid	
B. impatiens	52	1 queen	June 6, 1920	Colony successfully reared	
B. centralis	53	2 queens	June 9, 1920	Two large egg-cells made, but no eggs laid	
B. perplexus	54	1 queen	June 10, 1920	Colony successfully started	

for subsequent work along this line. In 1917, I secured four colonies out of fourteen experiments, or nearly twenty-nine per cent. Besides this, in four of the other fourteen experiments, the queens at one time or another gave evidence of some interest in the nest. In 1919, out of nineteen experiments the queens started colonies in thirteen of my artificial nests. This gave a successful percentage of seventy-seven plus in 1919. Two of the experiments of 1919 considered as failures should not really be so counted, for in one box one queen was killed and the other died of a nematode infection, and in the other instance both queens escaped. Out of thirteen experiments in 1920, ten must be considered as successful, or seventy-seven per cent. This same year, in two of the other three experiments considered as failures, the queens went so far as to make egg cells, and another colony might have been started if at that time it had been possible to continue these experiments longer in the spring. A survey of Table I. will show also that in 1917, 1919 and 1920 there were ten experiments in which but one queen was placed in each artificial nest. Colonies were started in seven of these ten experiments. The queens in two of the other three experiments of this nature never manifested any interest in the nest. One of the three, however, at one time took an interest in the nest, but finally abandoned it without laying eggs. All in all, I secured as good results, in proportion to the number of experiments, when one queen was used as when there were two.

SUMMARY.

Summarizing, I find that it is possible to induce the queens of certain species of North American bumblebees to start colonies in confinement when either one or two queens are used in the experiments. Plath (1923) achieved similar results in 1922 by the confinement of a single queen assisted by "one to three workers." In 1919 and 1920, in starting colonies under controlled conditions an average success of over seventy per cent. was attained. These results are indicative that the style and make-up of the artificial nest used in 1919 and 1920, as well as the procedure followed, is the most likely to produce results of any method thus far described. Furthermore, for the first time normal colonies of bumblebees were obtained by confining in an artificial nest a single queen unaided by introduced workers.

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STUDIES ON *PARAMECIUM.*

III. The Effects of Vital Dyes on *Paramecium caudatum.**

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The Protozoa are in many respects more suited for a study of the action of vital dyes on protoplasm than are Metazoan tissues, since with unicellular animals it is possible to determine readily a change in the normal life of the cell as well as the exact moment of death or recovery. The protoplasm of *Paramecium* may be used as a means of studying any selectivity towards the entrance of various vital dyes, as well as the affinity of the dye for different elements in the cell. On the other hand, caution should be observed in applying results obtained in this group of animals to any other group without adequate confirmatory evidence.

Material and Methods.

The animals used belonged to a "conjugating" clone of *Paramecium caudatum* (Race 385–1, Ball, 1925), which has been maintained in culture for over four years. The dyes were obtained from the various sources indicated in Tables I. and II. Usually, the stock solutions were made up with distilled water, in such concentrations that only a very small volume of dye solution was added to a relatively large volume of culture fluid containing the *Paramecium.* However, those dyes in which the lethal concentration was only slightly less than the maximum solubility were made up in the culture medium itself, since ordinary distilled water is usually fatal to these animals. The organisms were freed from the stain by centrifuging and washing them in *Paramecium*-free hay infusion drawn from the stock cultures.

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RESULTS.

Certain dyes did not stain the cytoplasm of normal living *Paramecium* (Table I.). Of the colors tested, these were anilin blue, alizarin, erythrosin, orange G, Lyons blue, purpurin, eosin Y, trypan blue, trypan red, and congo red; all of them except Lyons blue belong to the acid group of dyes. With these stains, color became visible in the cytoplasm only when the animals were dead or moribund. Dead animals usually stained almost immediately even though the cell membrane was not broken. The macronucleus of the dead organisms was stained by all of the dyes except erythrosin and orange G. In some instances, the cytoplasm might stain before the animal was entirely dead, but such paramecia were always swollen and sluggish, and very obviously moribund. Except in purpurin or in eosin, the *Paramecium* could live normally and apparently indefinitely in any of the above dyes as long as the cytoplasm did not stain.

TABLE I.

DYES NOT STAINING CYTOPLASM OF NORMAL LIVING *Paramecium.*

Dye.	Range of Concentrations.	Toxicity at Maximum Concentration. Estimated Per Cent. Dead in:	
		1 Hour.	12 Hours.
Anilin blue (N.)	1/90,000–1/55	0	5
Alizarin (M.)	1/90,000–1/16,000	0	60
Erythrosin (C. & B.)	1/90,000–1/5,000	80	100
Orange G (M.)	1/90,000–1/110	80	100
Lyons blue (C. & B.)	1/90,000–1/55	5	100
Purpurin (G.)	1/200,000–1/90,000	80	100
Eosin Y (G.)	1/90,000–1/55	100	—
Trypan blue (N.)	1/90,000–1/155	0	0
Trypan red (N.)	1/90,000–1/155	0	0
Congo red (M.)	1/90,000–1/110	3	5

(C. & B.) = Coleman and Bell Co.
(G.) = G. Grübler and Co.
(M). = H. A. Metz and Co.
(N.) = National Aniline and Chemical Co.

Purpurin, which has been used as an indicator for calcium in dead *Paramecium* (Sampson, 1925), does not stain any part of the living animal in acid or in alkaline solution. It does produce a peculiar blistering of the pellicle, which, in the majority of the organisms, first appears at the anterior end. Somewhat later, blisters occur laterally and posteriorly, although in a few paramecia, they may arise at the posterior end before or at the same time that they appear anteriorly (cf. Child and Deviney, 1926).

Eosin did not stain the living *Paramecium* at any concentration, nor did it stain the dead organisms for several hours at a strength of 1 to 55 up to 1 to 200, even though the cell-membrane had burst and the animals were cytolized. At a greater dilution—1 to 300 or more—the cytoplasm and the macronucleus quickly took up the stain.

Trypan blue or trypan red did not stain the cytoplasm of living *Paramecium* up to a concentration of 1 to 155, practically the maximum concentration possible. These two dyes, when ingested, will color the food vacuoles but not the cytoplasm, the vacuoles destaining, however, within three hours after the organisms are removed from the dye. According to Rohde (1917), trypan red stains *Paramecium* in an acid medium, but the animals of this clone did not stain with trypan red or trypan blue either in an acid or in an alkaline solution. Becker (1926) could not stain *Opalina* in trypan blue or in trypan red 1 to 10,000 or 1 to 20,000.

Although congo red did not stain the living cytoplasm, the food vacuoles took up the stain readily, even at a dilution of 1 to 300,000.

Table II. is a record of those dyes staining living *Paramecium;* they all belong to the basic group. Of these, the most suitable are bismarck brown, methylene blue, methylene green, neutral red, and toluidin blue. Except in highly toxic concentrations, anilin yellow and methyl violet stain the cytoplasm only very lightly, the color disappearing rapidly after the animals are removed from the dye. Safranin and basic fuchsin stain the cytoplasm only at strengths which are rather highly toxic, although the latter stains the contents of the food vacuoles very deeply in non-toxic concentrations.

With the exception of bismarck brown, all of the dyes employed in sufficient concentrations to stain the cytoplasm were eventually

TABLE II.

DYES STAINING CYTOPLASM OF NORMAL LIVING *Paramecium.*

Dye.	Range of Concentrations.	Toxicity at Maximum Concentration. Per Cent. Dead in 1 Hour.	Lowest Concentration at Which Cytoplasm Stains.	Toxicity at Lowest Concentration at Which Cytoplasm Stains. Estimated Per Cent. Dead in:		Number of Hours Required for Cytoplasm to Destain.
				1 Hour.	12 Hours.	
Bismarck brown (M.)	1/750,000–1/5,000	100	1/150,000	0	5	7
Methylene blue (C. & B.)	1/1,150,000–1/5,000	100	1/100,000	5	60	7
Methylene green (C. & B.)	1/1,150,000–1/5,000	100	1/37,500	5	100	4
Neutral red (N.)	1/2,250,000–1/5,000	100	1/150,000	3	70	9
Toluidin blue (C. & B.)	1/2,250,000–1/5,000	100	1/105,000	5	70	9
Basic fuchsin (C. & B.)	1/500,000–1/5,500	100	1/25,000	30	100	9
Safranin (E. & A.)	1/100,000–1/6,600	100	1/9,000	30	100	$1\frac{1}{2}$
Anilin yellow (U. C.)	1/125,000–1/3,300	100	1/5,500	0	5	1
Methyl violet (B. & W.)	1/3,000,000–1/100,000	100	1/500,000	20	90	2
Janus green B (N.)	1/450,000–1/45,000	100	1/180,000	40	100	7

(B. & W.) = Burroughs Wellcome and Co.
(C. & B.) = Coleman and Bell Co.
(E. & A.) = Eimer and Amend.
(M.) = H. A. Metz and Co.
(N.) = National Aniline and Chemical Co.
(U. C.) = Chemistry Laboratory, University of California, Southern Branch.

fatal to *Paramecium*. Some of the animals were capable of living at least six days, and apparently indefinitely, in a dilute solution (1 to 125,000) of bismarck brown, the cytoplasm staining a light brown, the food vacuoles taking a deeper color. Although the macronucleus could be stained while the animal was still actively swimming (neutral red and Janus green B), in no instance was an animal so stained observed to recover.

A dye was concentrated in the food vacuoles of *Paramecium* and stained their contents at a strength very much below that required to stain the cytoplasm. After the animals were removed from the dye, the food vacuoles retained their color long after the cytoplasm had destained. This was due also, in part, to the ingestion of stained bacteria, which had been thrown down by the centrifuge and transferred along with the *Paramecium*. It was noted particularly in solutions of neutral red, where the food vacuoles continued to stain for two days after the animals had been removed from the dye to fresh infusion. Although nothing in the medium was visibly stained, the contents of the food vacuoles took on a pink color as the latter formed at the base of the gullet, and became a deep red as the vacuole began passing forward from the posterior end of the animal. Any observation of the time required for destaining *Paramecium* should be based on the behavior of the cytoplasm and not of the food vacuoles, which may retain their color until nearly all of the bacteria in the medium have been digested.

With all of the stains used, the color of the cytoplasm disappeared fairly rapidly after the organisms were removed from the dye solution, in no case being detectable for more than nine hours, and in many instances fading out much sooner. The time required for destaining the cytoplasm was independent both of the length of time it had been stained and of the concentration of the dye used. If the stained animals were removed to a dilute solution of the dye, their life was prolonged, but they were eventually killed unless the cytoplasm destained. It is possible that the results of Baldwin (1920), in which paramecia so treated lived for more than eight days may have been based on the retention of the dye in the food vacuoles rather than in the cytoplasm proper.

The comparatively rapid loss of the color by stained paramecia indicates the ability of the protoplasm of *Paramecium* to reduce or to eliminate the dye in a few hours; or perhaps even marks a complete change of protoplasm in this time.

A solution of 1 to 180,000 Janus green B shows almost immediately a number of small rod-like bodies, probably mitochondria (Causey, 1926; Hogue, 1926). These were somewhat more widely scattered through the cytoplasm than those shown in Causey's figures, although they could also be detected between and around the food vacuoles. The dye is exceedingly toxic for *Paramecium*, killing them in a few hours at a concentration as low as 1 to 450,000. The cytoplasm stains bluish green at 1 to 180,000, the macronucleus taking on a yellow color before the animal dies. The food vacuoles are at first bluish green, later becoming light pink due to the reduction of the Janus green B to diethylsafranin.

All of the above experiments were performed in the diffuse light of the laboratory, the cultures being kept under glass, out of the direct sunlight. Animals stained with certain dyes (Table III.) give a very striking reaction to strong light. These stained *Paramecium* drawn from a flourishing culture soon become sluggish and settle practically motionless on the bottom of the Syracuse watch glass. They can be observed in this condition under a compound microscope with the iris diaphragm almost completely closed, and a heat screen interposed between the organisms and the source of light. If the diaphragm now be suddenly opened, practically all of the animals begin darting violently about, giving the avoiding reaction and soon swimming out of the field. The reaction begins in from four to five seconds after opening the diaphragm, and occurs almost simultaneously in 90 to 95 per cent. of the animals exposed. If the *Paramecium* are observed under a 16 mm. objective, the field will be entirely empty in two to three minutes, even though several hundred motionless animals may have been present previously. If the diaphragm be closed before the field is completely vacant, the remaining organisms settle to the bottom of the dish again within one minute. If only the food vacuoles are stained but not the cytoplasm, the animals do not exhibit these reactions to light. The uniformity in reaction-time is evidently due to the fact all the *Paramecium* belonged to a single clone.

TABLE III.

EFFECT OF LIGHT ON TOXICITY OF DYES FOR *Paramecium*.

Strength of Dye Used.	Number of Hours Required to Kill in:		Light Reaction.
	Strong Light.	Darkness.	
Eosin Y (G.) 1/250	19 (95% dead in 3 hours)	Lives indefinitely	+
Methylene blue (C. & B.) 1/100,000	1	24	+
Methylene green (C. & B.) 1/45,000	2	17	+
Neutral red (N.) 1/125,000	1	25	+
Toluidin blue (C. & B.) 1/107,000	1	48	+
Bismarck brown (M.) 1/125,000	Lives indefinitely	Lives indefinitely	–
Basic fuchsin (C. & B.) 1/18,000	2½	2½	–
Trypan blue (N.) 1/1,500	Lives indefinitely	Lives indefinitely	–
Trypan red (N.) 1/1,500	Lives indefinitely	Lives indefinitely	–

Furthermore, those stained paramecia exhibiting this light reaction die much sooner when exposed to strong light than when kept in the dark. Table III. shows the difference in survival-time between the two groups of organisms. An ordinary Mazda lamp served as the source of light. The dishes containing the organisms were covered with water-filled glass containers and exposed to a current of air from an electric fan. The temperature in the two series did not differ by as much as a degree during the course of the experiments. Animals stained in bismarck brown, basic fuchsin, trypan blue, or trypan red showed no change in behavior upon sudden illumination; correspondingly, the toxicity of these dyes was not increased in the light. While living *Paramecium* did not stain in eosin, and although a concentration as great as 1 to 250 was not toxic for them as long as they were kept in the dark, they had a marked avoiding reaction to light and died rapidly under strong illumination. Raab (1900) studied the greater toxicity of *fluorescent* dyes for infusoria, and similar results were recorded by Pereira (1925), who tested the action of eosin on the germ cells and larvæ of *Arbacia*.

According to Packard (1925), light increases the permeability of the protoplasm of *Paramecium;* the greater toxicity is not solely a matter of permeability, however, since such stains as neutral red penetrate the cytoplasm almost immediately, yet the animals live twenty times as long in the dark as under strong illumination. Child and Deviney (1926) noted greater toxicity of eosin, neutral red, and methylene blue for *Paramecium* upon exposure to light, while Baldwin (1920) found that stained paramecia were more susceptible to X-rays than unstained ones. In the clone used in the present experiments, long contact with the dye does not produce any acclimatization on the part of the *Paramecium;* animals kept in the dark in neutral red 1 to 125,000 or in methylene blue 1 to 100,000 for eighteen hours die just as soon under strong illumination as those exposed to the light and the stain simultaneously. Furthermore, merely subjecting the dye to intense light does not make it more toxic when subsequently added to cultures of *Paramecium.*

Discussion.

The literature on vital staining in the Protozoa has been well reviewed by Nirenstein (1920) and by Vonwiller (1921), and consequently will not be considered here.

All of the dyes which stained the cytoplasm of normal living *Paramecium* belonged to the basic group. None of the acid dyes stained the cytoplasm of normal animals, although most of them stained the dead or dying organisms. Lyons blue was the only basic dye not staining the living animals. There have been many different interpretations of the meaning of the reactions taking place between different dyes and living protoplasm (Nirenstein, 1920). If it be concluded from the above results that, under normal conditions, the protoplasm of *Paramecium* is acid in reaction, this would confirm Crozier's observation (1923), using brom thymol blue as an indicator, that the pH of the living protoplasm of *Paramecium* is 6.7 or less, and not alkaline or neutral as is usually maintained.

The period required for destaining the organisms was independent both of the length of time necessary for staining them, and of the concentration of the dye used. Furthermore, if the cytoplasm stained at all, the color was as intense with a dilute as with a concentrated solution of the stain. In dilute solutions, the cytoplasm or the food vacuoles might stain deeply, yet the fluid medium or the bacteria might show only a very slight color. According to Nirenstein (1920), the capacity of the living cell for taking up dyes depends upon the distribution of lipoids in the protoplasm. It may possibly mark merely a more intense staining reaction rather than an increased concentration of the dye. The failure of the acid dyes to stain the cytoplasm does not prove that they fail to enter as freely as do the basic ones; the behavior of the organisms indicates rather that the dyes of the acid group do penetrate the cell without staining the cytoplasm.

Certain dyes act as sensitizers for the destructive action of light; their toxicity is not increased by exposure to strong light before addition to the *Paramecium* cultures. The effect is not solely one of greater permeability since the shorter length of life in light bears no relation to the ease of penetration of the dye. With this clone of *Paramecium*, the harmful effect of light for the stained organisms began within five seconds of exposure, as indicated by the behavior of the animals themselves.

This work on vital stains was started originally in the hope of employing it to mark individuals belonging to different clones of *Paramecium*. Thus, it would be possible to determine whether or not extra-clonal conjugation can be obtained under experimental conditions. However, the rapidity with which the cytoplasm of these animals loses its color after being removed from the stain precludes the use of this method as a means of identification. It seems probable that we shall have to employ morphological differences, other than those of size, such as the notched mutant recently described by Dawson (1926).

Conclusions.

1. The only dyes staining the cytoplasm of normal living *Paramecium* belonged to the basic group. Those found to be most suitable were bismarck brown, methylene blue, methylene green, neutral red, and toluidin blue.

2. The cytoplasm of normal animals could not be stained by

any of the acid dyes used, although these might stain the contents of the food vacuoles, or the cytoplasm of dead or dying *Paramecium.*

3. Animals with stained cytoplasm could live indefinitely in a dilute solution of bismarck brown; in all of the other dyes used, the stained paramecia eventually died unless they were removed from the stain. The presence of the dye in the food vacuoles but not in the cytoplasm was not necessarily fatal to *Paramecium*.

4. Although the macronucleus could be stained while the animal was still alive, such animals eventually died even though removed from the stain.

5. The stain disappeared from the cytoplasm of the living animals within a few hours after their removal from the dye solution.

6. The exposure to light of *Paramecium* having the cytoplasm stained by certain dyes, or of *Paramecium* in a concentrated solution of eosin, produced a marked avoiding reaction within five seconds. The animals giving this reaction died much more rapidly in strong light than in darkness.

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BIOLOGICAL BULLETIN

THE ZOÖCHLORELLÆ OF *FRONTONIA LEUCAS.*

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INTRODUCTION.

It is common knowledge that many organisms of the animal kingdom harbor within their bodies various forms of algæ. Dangeard ('02) writes at length on the probable relation of the zoöchlorellæ, in *Paramecium bursaria*, to their host. The experiments of Lipska ('10) on a culture of *Paramecium caudatum* which contained green algæ are also worthy of note. Geza Entz ('81–2) presents evidence which he thinks is indicative of a symbiotic relation between *Stentor polymorphus* and green algæ. Bary ('79) was the first investigator to allude to the relation between the zoöchlorellæ and their host as a symbiotic one. But Dangeard ('02) says that the relation is incompletely proven, and Bouvier adds that we have not a definite example demonstrating the usefulness of the algæ to their host.

The work of this paper was conducted in an effort to add further knowledge on the relation of endoplasmic algæ to their host. The host used in these experiments has been identified as *Frontonia leucas* Ehrenberg.

I wish to express my gratitude to Dr. B. D. Reynolds, under whose direction this work was done, for reading the manuscript and making a number of important suggestions. I am also indebted to Dr. W. A. Kepner and Mr. J. B. Looper for helpful advice and criticisms.

DISAPPEARANCE OF ZOÖCHLORELLÆ FROM HOST.

In *Frontonia* taken from a collection made near the University of Virginia, from a very small stagnant pool, on June 23, 1925, the zoöchlorellæ were innumerable. Artificial cultures were attempted with little success. For this reason collections were kept in the laboratory just as they were when taken from the pool, except that fresh spring water was added daily.

After thirty days specimens from these cultures showed a decided decrease in the number of zoöchlorellæ. Fresh collections were then made and treated as before with like results. It was found that with such treatment *Frontonia* would, in from thirty to forty days, be partially if not entirely free of zoöchlorellæ.

On September 15, 1925, seven cultures in fresh spring water[1] were started, using Syracuse watch glasses as containers. Twelve specimens were placed in each culture, and fresh spring water was added daily. By September 18 approximately fifty per cent. of the total number of the specimens had disappeared. September 21, all specimens had disappeared from five cultures.

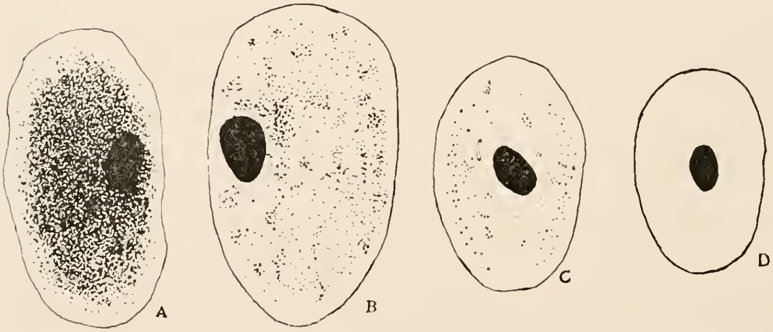

FIG. 1. Camera lucida drawings. × 375. *A*, normal specimen taken on date of collection. *B*, after twelve days in fresh spring water. *C*, after twenty-five days in fresh spring water. *D*, after forty days in fresh spring water.

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[1] Analysis of the spring water was made by Mr. R. K. Witt, under the direction of Dr. John H. Yoe of the Department of Chemistry, University of Virginia, and showed the following:

	Parts Per Million.
Total solids	37.6
Fixed residue	28.0
Loss on ignition	9.6
Organic matter	Very small amount as indicated by a slight charring on ignition.

The pH value of the spring water was fairly constant at 7.2.

Three living specimens were found in one, and five in the other. On September 22 duplicate cultures were started with similar results.

September 30, collections of *Frontonia* harboring innumerable zoöchlorellæ were made and change from the original culture medium to fresh spring water was made gradually by drawing off a part of the medium daily and replacing it with fresh spring water. Under these conditions it was observed that within forty days the zoöchlorellæ had completely disappeared, while the ciliates showed no signs of death.

Slides made at intervals extending over the forty-day period corroborate these observations (Fig. 1). Various stains and fixatives were used in making the slides, but the most satisfactory results were obtained from Looper's fixative[1] and iron hæmotoxlyn.

From these observations it is evident that *Frontonia* may be freed from their zoöchlorellæ by a gradual transference from their natural habitat to a medium of fresh spring water.

Increase in Number of Zoöchlorellæ in Host.

In a collection made from a large semi-stagnant pond, July 2, 1925, *Frontonia* with very few zoöchlorellæ were abundant.

MARINE BIOLOGICAL LABORATORY
WOODS HOLE
LIBRARY

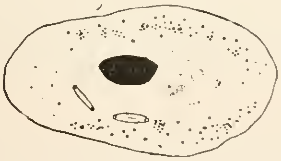

FIG. 2. Typical specimen from a collection in which the *Frontonia* contained but few zoöchlorellæ.

Slides from the collection showed the average specimen to contain from fifty to one hundred zoöchlorellæ (Fig. 2). The collection was set aside and remained untouched until August 1. On this date the zoöchlorellæ in twelve specimens examined were innumerable (Fig. 3).

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[1] Make a thin smear of Mayer's egg albumen the size of a dime on a clean slide. Place the ciliates to be fixed on the smear in a drop of water just large enough to make a thin layer over the albumen. Invert the slide over the mouth of a small bottle containing a mixture of equal parts of 95 per cent. alcohol, glacial acetic acid and formalin. Let the slide remain in this position 30 seconds to a minute. Remove the slide and slant it back and forth until the film of water has almost disappeared. Wash gently in water and stain.

On June 8, 1926, eight cultures, containing twelve specimens per culture, of *Frontonia* harboring relatively few zoöchlorellæ, were started in fresh water to which a small amount of detritis from the collecting dish was added. These cultures remained in the laboratory with abundant light, but never in direct sunlight, until July 8, without adding more fresh spring water. At this time slides were made from each culture. The zoöchlorellæ in 95 per cent. of the animals were innumerable. Checks which corroborate these findings were made with cultures started June 10 and 12.

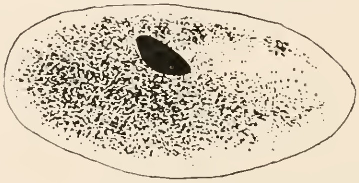

FIG. 3. Showing increase of zoöchlorellæ in *Frontonia* after the same collection (Fig. 2) had remained in the laboratory for thirty days.

From these results the conclusion, that the number of zoöchlorellæ in *Frontonia leucas* may be increased by an increase in stagnation and putrefaction of the medium in which they live, seems logical.

INFLUENCE OF OSMOTIC PRESSURE.

In an effort to explain, beyond the simple statement of the difference between stagnant and fresh water, these variations in the number of zoöchlorellæ inhabiting each host, cultures with various osmotic pressures were made as follows: Three cultures using distilled water, three cultures using fresh spring water, three cultures of one per cent. dextrose in spring water. Into each of these cultures were placed six specimens in which the zoöchlorellæ were numerous, and six with no zoöchlorellæ. It was found that upon being put into distilled water the organisms with zoöchlorellæ promptly disintegrated. Ejection of zoöchlorellæ, desmids, and diatoms preceding death only by a few moments. The organisms which contained no zoöchlorellæ often lived several hours. In fresh spring water specimens with zoöchlorellæ lived from five to eight days. Specimens without zoöchlorellæ lived indefinitely. In one per cent. dextrose specimens with zoöchlorellæ lived eight days or more. Specimens without zoöchlorellæ averaged two days. It was found also that if the cultures in one per cent. dextrose were placed in fresh media daily, specimens with zoöchlorellæ lived indefinitely, while those without lived four or five days.

Experiments begun June 24, 1926, showed that *Frontonia* with abundant zoöchlorellæ may be induced to live several days in a three per cent. solution of dextrose by beginning at one half per cent., changing the medium daily, and increasing the concentration one half per cent. every fourth day. But under no condition have specimens without zoöchlorellæ been induced to live more than a few hours in a medium exceeding one per cent.

In two cultures containing one per cent. dextrose vegetative reproduction was observed in *Frontonia* harboring zoöchlorellæ where fermentation had been going on three and four days. *Frontonia* without zoöchlorellæ would not live in a medium of this kind.

These observations suggest that specimens harboring zoöchlorellæ are more resistant to media of greater osmotic pressure than are free specimens.

HYDROGEN ION CONCENTRATION.

It has been observed that as fermentation progresses in dextrose cultures the hydrogen ion concentration increases; *e.g.*, a one per cent. solution with a pH value of 7.2 made July 18 showed, 24 hours later, a pH of 7.1. After 48 hours the pH was 7.0, after 72 hours 6.9, and on the fourth day it had become 6.7. Observations made on one, two, and three per cent. solutions showed similar increases in hydrogen ion concentration during fermentation.

The hydrogen ion concentration of media from natural collections was then studied. Data gathered from this study are given in Table I.

TABLE I.

SHOWING HYDROGEN ION CONCENTRATION OF THREE COLLECTING POOLS OVER AN EXTENDED PERIOD OF TIME.

Date	August, 1925.							June and July, 1926.							
	1	4	7	10	13	15	17	12	15	18	21	24	27	30	8
A...	7·4	7·4	7·3	7·3	7·4	7·5	7·5	7·3	7·3	7·3	7·4	7·4	7·4	7·5	7·5
B...	7·4	7·3	7·3	7·3	7·4	7·4	7·4	7·3	7·3	7·2	7·2	7·3	7·3	7·4	7·4
C...	7·2	7·2	7·1	7·1	7·1	7·2	7·2	7·1	7·1	7·0	7·0	7·2	7·2	7·3	7·4

A from pool containing *Frontonia* with no zoöchlorellæ.

B with relatively few.

C in which the zoöchlorellæ were innumerable.

Beginning June 20th there were increasing rains until July 10. On July 12 collections were taken from all pools where *Frontonia* had previously been found and a uniform scarcity of zoöchlorellæ was observed.

As stated above, when specimens containing relatively few zoöchlorellæ are placed in cultures and left undisturbed, so that the culture medium becomes somewhat stagnant, the zoöchlorellæ increase very noticeably. Also that when specimens containing numerous zoöchlorellæ are changed from their original culture to a medium of fresh spring water, the zoöchlorellæ decrease very noticeably. There is correlated with this increase and decrease in the number of zoöchlorellæ, an increase and decrease in the hydrogen ions. The following examples are characteristic of the finding in this respect.

A collection in which the *Frontonia* showed relatively few zoöchlorellæ had a pH value of 7·3. This culture remained in the laboratory and the hydrogen ion concentration was taken every other day until the specimens harbored innumerable zoöchlorellæ, thirty days. Data thus obtained are given in Table II.

TABLE II.

SHOWING VARIATIONS IN pH VALUE OF THE MEDIUM AS THE NUMBER OF ZOÖCHLORELLÆ INCREASED.

	June and July, 1926.													
Date.....	10	12	14	16	18	21	24	27	30	3	6	10	14	18
pH......	7·3	7·3	7·2	7·0	6·9	6·9	6·7	6·7	6·8	6·8	6·9	7·0	7·0	7·1

A collection in which the *Frontonia* showed innumerable zoöchlorellæ had a pH value of 6.8. This culture remained in the laboratory and a change from the original culture medium to fresh spring water was made gradually. The hydrogen ion concentration was taken every other day until the specimens harbored few, and in some cases no zoöchlorellæ. Data thus obtained are given in Table III.

TABLE III.

SHOWING VARIATIONS IN pH VALUE OF THE MEDIUM AS THE NUMBER OF ZOÖCHLORELLÆ DECREASED.

June and July, 1926.

Date.....	8	10	12	14	16	18	20	22	25	28	30	4	7	10
pH......	6.8	6.8	6.9	6.9	6.9	7.0	7.0	7.0	7.1	7.2	7.2	7.2	7.3	7.3

DISCUSSION.

An apparently significant fact observed during the course of these experiments is: That *Frontonia* with abundant zoöchlorellæ refuse to live in media of clear, fresh water. They seem to require a stagnant, even putrid, medium containing detritis, which serves as a place of hiding, much as do *Spirostomum ambiguum*. Indeed these two protozoans have a common habitat in every instance within the experience of the writer. But *Frontonia* without zoöchlorellæ have been found in great abundance in fresh running streams.

It will be noticed from the foregoing investigations that a host may experience either an increase or a decrease in the number of its zoöchlorellæ, but that no statement is made concerning their ability to regain them after having been completely freed. Regarding this subject we may say that repeated efforts to induce *Frontonia* with no zoöchlorellæ to acquire them were unsuccessful. Dangeard says of the zoöchlorellæ of *Paramecium bursaria* that they do not live long when freed in the medium of their host. This suggested the possibility of inducing *Frontonia* without zoöchlorellæ to appropriate them directly from the body of a crushed specimen. Efforts to demonstrate this, however, were unsuccessful.

Another feature that seems worthy of further investigation may be observed in the camera lucida drawings given in Fig. 1; *e.g.*, the evident decrease in the size of the host as the zoöchlorellæ disappear. Minchin quotes Popoff as observing these variations in size of *Frontonia leucas* and crediting them to a difference in temperature of the media in which the *Frontonia* live.

The experiments of this paper were conducted in a laboratory where both large and small, infected and uninfected *Frontonia* were exposed to the same conditions of temperature. Yet differences of as much as 100 microns have been found in specimens taken from the same culture, and these variations have persisted throughout the study. Therefore it seems unlikely that temperature could have had any part in this variation.

Of fifty specimens, without zoöchlorellæ, measured, the average length was 225 microns, while the nucleus averaged 16 microns in length. Of fifty specimens, with innumerable zoöchlorellæ, measured, the average length was 310 microns and the nucleus averaged 18 microns in length.

These observations tend to demonstrate that a mass relation exists between cytoplasm and nucleus. Similar nucleoplasmic relations have been demonstrated in different species of *Arcella* by Hegner (1920) and Reynolds (1923).

Lipska (1910) found that when placed in the dark, *Paramecium caudatum* with zoöchlorellæ would live eight days or more, that *Paramecium* without zoöchlorellæ died between the second and fourth days, and concluded that the zoöchlorellæ were responsible for the greater resistance shown by the *Paramecium* with zoöchlorellæ, in that they furnished a supply of oxygen.

The following experiment was carried out with *Frontonia leucas:* Specimens with zoöchlorellæ were placed in darkness, in their original culture medium, and exposed to the same conditions of temperature as before, but no appreciable difference was noticed in their death rate as compared with those in the light. This, however, does not preclude the possibility of the host obtaining oxygen from the zoöchlorellæ, because in darkness, where photosynthesis is impossible, zoöchlorellæ cannot give off oxygen.

In his work on the zoöchlorellæ of *Paramecium bursaria*, Dangeard found that starch granules were abundant in the zoöchlorellæ and it was his belief that the host appropriated these starch granules as food, though he was unable to demonstrate this.

In this work on the zoöchlorellæ of *Frontonia*, starch granules were found to be abundant in the zoöchlorellæ, but efforts to show that they were ever taken into the food vacuoles of the host have thus far been unsuccessful.

SUMMARY.

1. *Frontonia* with zoöchlorellæ require a medium of greater stagnation and putrefaction than *Frontonia* without zoöchlorellæ.
2. *Frontonia* may be freed from their zoöchlorellæ by a gradual transference from their natural habitat to a medium of fresh spring water.
3. The number of zoöchlorellæ in *Frontonia* may be increased by increasing the degree of stagnation and putrefaction of the medium in which they live.
4. *Frontonia* harboring zoöchlorellæ are more resistant to media of greater osmotic pressure than are free specimens.
5. The difference between the hydrogen ion concentration of media in which *Frontonia* with innumerable zoöchlorellæ are found and media in which *Frontonia* with no zoöchlorellæ are found, averages 0.3.
6. With an increase in the number of zoöchlorellæ there is a corresponding increase in the hydrogen ions, and conversely.
7. The ability of *Frontonia* harboring zoöchlorellæ to live in a greater concentration of dextrose than *Frontonia* without zoöchlorellæ is correlated with a greater hydrogen ion concentration caused by fermentation of the dextrose.
8. Efforts to induce *Frontonia* without zoöchlorellæ to gain them were unsuccessful.

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GYNANDROMORPHS AND OTHER IRREGULAR TYPES IN *HABROBRACON.*

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In a series of papers and abstracts on the genetics of the parasitic wasp, *Habrobracon*, mention has been made of various irregular types not falling in line with the general principle of sex-linkoid inheritance.

These cases are considered to be of fundamental significance from the point of view of maturation and cleavage. They include impaternate females, mosaic males and females, gynandromorphs, and intersexual males.

Among the higher Hymenoptera it is now generally assumed that females arise from fertilized eggs, males from unfertilized. Exceptions to this have, however, been claimed in certain ants (Wheeler, W. M., 1903). Among the lower Hymenoptera conditions are much more irregular. Some species of saw-flies are exclusively or predominantly "female-producing," thelytokous; others "male-producing," arrenotokous; while others, amphitokous, produce both sexes parthenogenetically.

Gall wasps with their alternation of sexual and parthenogenetic generations have in the latter both male-producing and female-producing females.

Polyembryonic Chalcidoids produce male broods from unfertilized eggs, female broods from fertilized, but Patterson (Patterson, J. F., 1917) has shown that certain broods, predominantly female, may contain a few males, presumably derived from mitotic irregularities in the polygerm.

Among the Ichneumonoids, to which group *Habrobracon* belongs, arrenotoky is the rule but some species are thelytokous. In a species that is predominantly arrenotokous it has been shown (Hunter, S. J., 1909, and Webster, F. M., 1909) that the tendency to produce females is probably inherited.

The irregular forms discussed in the present paper have occurred in *Habrobracon juglandis* (Ashmead) with the exception of gynandromorphs, freaks 269 and 271, and intersexual males, freaks 193, 273, 274, and 275. The latter were found in the European species, *Habrobracon brevicornis* (Wesmael).

IMPATERNATE FEMALES.

In the stock of *Habrobracon juglandis* (Ashmead) from Lancaster, Pennsylvania, females have been, with one very doubtful exception, derived from mated females. In material from Iowa City and in descendents of crosses between the Lancaster (L.) and Iowa City (I.) materials, undoubted cases of thelytoky have occurred.

Females are exposed to males either in culture vial *a* or previously after which they are ordinarily separated. The females are then passed through vials *b*, *c*, etc. Relatively more daughters are produced in the earlier vials. The supply of sperm seems to be exhausted after a length of time varying in individual cases. When female ratios per brood are extremely low the few females usually occur in vial *a*. This applies for example to the daughters of patroclinous diploid males. Single females occurring in later vials may, therefore, be suspected of being impaternate unless other conditions preclude the possibility.

In individual culture counts of the pure I. material, thirty-seven virgin females produced only males, 3,288. Thirty-one females that had been exposed to males produced only males, 3,746. Sixty-three females exposed to males produced both males, 2,933, and females, 2,719. Female percentages in these sixty-three cultures ranged from 18 to 74.

Besides the bisexual broods above recorded there were some in which but a single female appeared.

In one case (April, 1923) a female which was observed to mate with a single male produced 142 males and in vial *c* a single female.

In another case (April, 1923) a female which had been exposed to males produced 97 males and in vial *e* a single female. Each of these two cases is aberrant in that only one female appeared and she was produced relatively late in the mother's life. They are, however, inconclusive as regards thelytoky since the mothers had been exposed to males.

Two cases of undoubted thelytoky occurred in the pure I. material. In one (December, 1922) a virgin female produced 97 males and in vial *b* a single female, freak 277. In the other (April, 1923) a virgin female produced 97 males and in vial *c* a single female, freak 278.

Freak 276.—A sooty (mesosternum) female from I. stock 11 ($OD_{II}s_{II}$) was mated to an orange (eye), defective (vein r_4) male, $od_{II95}S_{II}$, derived from L. stocks 6 and 10 and I. stock 11. There were produced males—type 1, sooty 49, and females—type 87, grading to sooty 8, and defective 1. One of these type females produced males—type 7, sooty 29, defective 29, sooty defective 4, orange 4, orange sooty 29, orange defective 28, and orange sooty defective 13, showing free segregation of orange and linkage of sooty and defective. In vial *f* an orange female, freak 276, was found (April, 1923). No males appeared. Since the F_1 female had been exposed only to her black-eyed brothers her single orange daughter must have been impaternate. She produced males—orange 1, orange sooty 7, orange defective 5, orange defective sooty 8, and females—orange 3, orange sooty 3, orange defective 5. These numbers are not very satisfactory for such variable characters as sooty and defective. Moreover, L. and I. stocks possess minor factors which, when recombined, produce the characters sooty and defective without the factors s_{II} and d_{II95}.

It is assumed that thelytoky occurs by the suppression of the second oöcyte division. In this case the first division separated the tetrad into dyad OO which passed into the polar body, and oo which entered the cleavage nucleus.

Origin of Freaks 250, etc.—An orange, defective, wrinkled male of the twelfth inbred generation descended from the impaternate female, freak 276, was crossed with a sooty female from I. stock 11. There resulted in F_1 34 black males and 120 black females. Twenty-three of these females were isolated. Fifteen of them had evidently mated with their black-eyed brothers since they produced black-eyed females.

The F_2 generation consisted of males—type 217, sooty 240, defective 91, defective sooty 91, wrinkled and wrinkled defective 378, wrinkled sooty and wrinkled sooty defective 123, orange 193, orange sooty 168, orange defective 133, orange defective sooty 65, orange wrinkled and orange wrinkled defective 390, orange wrinkled sooty and orange wrinkled sooty defective 137, and females—type 900, sooty 16, defective 67, defective sooty 2, besides two impaternate females (freaks 257, 259), one mosaic male (freak 256), and three gynandromorphs (freaks 250, 255, 258) discussed in the present paper.

Disregarding the freaks we find among the total of 2,244 males, 1,086 or 48.39 ± 0.71 per cent. orange, 1,028 or 45.81 ± 0.71 per cent. wrinkled, and 824 or 36.72 ± 0.69 per cent. sooty. Orange is practically equal to expectation. Wrinkled is somewhat below due to death of the most extreme cases in cocoons. Sooty is low on account of method of grading.

Defectiveness was not determined in wrinkled wasps. Among the 1,216 with flat wings there are 398 or 32.73 ± 0.91 per cent. defective. This is lower than expectation when d_{II95} alone is involved although there is always some overlapping with normal. Probably modifiers are involved since linkage between wrinkled, w, and defective, d_{II95}, is absent. (Whiting, P. W., 1926*a*.)

Among the 1,216 flat-winged wasps there were 564 or 46.36 ± 0.96 per cent. sooty while among the 1,028 wrinkled there were 260 or 25.29 ± 0.91 per cent. sooty. The relation is contrary to expectation on a basis of linkage. The explanation is found in the fact that wrinkled wasps have lighter mesosterna on the average than flat-winged.

Although we know that factors, o, w, S_{II}, and d_{II95} were brought in by the male parent and O, W, s_{II}, and D_{II} by the female, no reliance can be placed upon the composition of any F_2 individual with respect to s_{II} and d_{II95} without adequate progeny test.

Freak 257.—One of the F_1 females, $od_{\text{II95}}wOD_{\text{II}}W$, mentioned under *Origin of freaks 250, etc.*, produced males—type 7, defective 5, wrinkled and wrinkled defective 6, orange 4, orange defective 3, orange wrinkled and orange wrinkled defective 6, and 110 black females besides an orange female (freak 257) with flat wings and normal r_4 which appeared in vial *e* (February, 1924). Since no orange females were to be expected we have here a case of thelytoky. Breeding test of this orange female showed her homozygous for normal venation, heterozygous for wrinkled.

Her orange-eyed sons were 35 with flat wings to 44 wrinkled. In 22 of the wrinkled in which r_4 could be observed it proved to be normal. The 57 normal demonstrate that d_{1195} was not present and this was evidenced also by later generations.

Freak 257 therefore originated from a first oöcyte division as follows:

$$\frac{\text{First polar body}\quad OOd_{1195}d_{1195}Ww}{\text{Cleavage nucleus}\quad ooD_{11}D_{11}Ww}$$

Freak 259.—A second F_1 female, $od_{1195}wOD_{11}W$, mentioned under *Origin of freaks 250, etc.*, was set and produced males—type 37, defective 10, wrinkled and wrinkled defective 36, orange 35, orange defective 18, orange wrinkled and orange wrinkled defective 41. In vial *h* an orange female (freak 259) appeared (March, 1924). This female had deformed gonapophyses and sting but was otherwise normal. No offspring could be obtained from it and hence its exact composition as regards d_{1195} and w is not known. Since it had flat wings and normal r_4 vein there was present at least one dose of W and probably of D_{11}. It demonstrates that for orange the first oöcyte division was reductional.

MOSAIC MALES AND FEMALES.

Five males with mosaic eyes are reported in the present paper. They are regarded as haploid mosaics from unfertilized eggs (haploid mosaic arrenotoky) in the formation of which both products of the second maturation division (oötids) have become cleavage nuclei. Maturation has been carried a step farther than in the case of impaternate females.

Freak 65.—Orange defective from the early L. material was bred up to L. type stock 1 for nine generations alternating with generations segregating orange and defective from the diheterozygous females. An orange defective male of the ninth segregating generation was mated to a type female, L. stock 1. An F_1 female produced males—type 11, defective 8, orange 4, orange defective 4, and females—type 2, besides a defective (grade 5) male (freak 65) with orange eyes and black ocelli, appearing in vial *b* (August 4, 1921). Wings and compound eyes of this freak must have been of maternal origin for his diheterozygous mother, $OoD_{11}d_{1195}$, had mated only with type brothers. Ocelli may have been either of paternal or of maternal origin. The male was absolutely sterile, although very active and willing to mate. It was kept alive forty days and mated to twenty-one females. There were produced no females among 757 males. Dissection showed testes to be unattached. There was a single vas deferens ending blindly on one side, none on the other. Penis and all external parts were normal.

While no evidence exists as to the origin of the black ocelli it may be tentatively assumed by analogy with succeeding cases that this male arose from an unfertilized egg. Oöcyte divisions may be represented as follows:

$$\frac{\text{First polar body}\quad OoD_{11}(D_{11})}{\text{Cleavage nuclei}\quad O(d_{1195})\,|\,o(d_{1195})}.$$

As regards orange the first division was equational, the second reductional. We know that d_{1195} remained in the egg for r_4 veins were both defective but other tissues might have had either D_{11} or d_{1195}. As regards defective therefore, the first division may have been either reductional or equational. Allelomorphs set in parentheses may be exchanged in the formula.

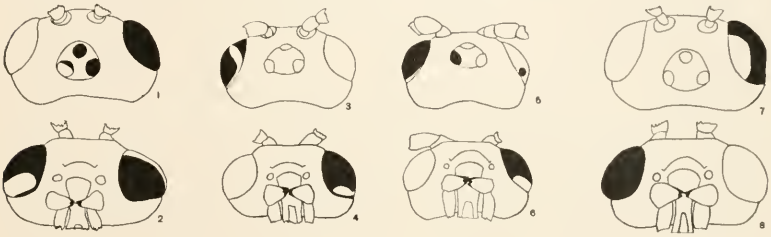
Freak 256.—An F_1 female, $od_{1195}wOD_{11}W$, of those mentioned under *Origin of freaks 250, etc.*, produced males—type 24, defective 8, wrinkled and wrinkled defective 20, orange 9, orange defective 8, orange wrinkled and orange wrinkled defective 14, and females—type 67, defective 8, and a male (freak 256) with normal r_4 vein, flat wings and mosaic eyes appearing in vial *f* (February 19, 1924). Eyes are shown in Figs. 5 and 6. The antennæ of this male had probably been injured by too much ether. It seemed impossible to obtain a mating. Histological preparation showed internal organs apparently normal.

His origin may be represented as follows:

$$\frac{\text{First polar body}\quad Ood_{1195}(d_{1195})w(w)}{\text{Cleavage nuclei}\quad O(D_{11})(W)\,|\,o(D_{11})(W)}.$$

As regards orange the first division was equational, the second reductional. Because of lack of adequate test, the case for wrinkled and defective is uncertain. Probably W and D_{II} remained in the egg as evidenced by the flat wings and normal r_4 vein of the specimen. In this case the first division may have been either reductional or equational for these factors.

Freak 154.—A black female heterozygous for orange, set with two orange brothers from mixed stock (I. x 5) produced males—16 black and 17 orange and females—18 black and 26 orange, besides a male, freak 154, with ocelli and right eye orange, left eye mixed. (February, 1923.) Figs. 3 and 4.



FIGS. 1–8. Dorsal and ventral views of heads of mosaic-eyed males of *Habrobracon juglandis*, showing distribution of black and orange elements. × 29. 1 and 2, freak 185. 3 and 4, freak 154. 5 and 6, freak 256. 7 and 8, freak 264.

Matings with nine orange females resulted in 664 orange males and 598 black females. Only black was therefore transmitted. Maturation of the egg producing this mosaic may be represented as follows:

$$\frac{\text{First polar body Oo}}{\text{Cleavage nuclei } \mathit{O} \,|\, \text{o}}.$$

Italics are used to denote origin of gonads.

Freak 185.—A defective (grade 2) sooty male of mixed ancestry (L. stocks 5, 6, 10, and I. stock 11) arose from a line lacking d_{II95} but having at least one minor factor, d_x, causing a small proportion of defectives to appear. This male, $Os_{II}d_x$, was crossed to a female of L. stock 5, $oS_{II}D_x$. A black-eyed virgin daughter produced males—type 45, defective 3, sooty 46, defective sooty 4, orange 43, orange defective 2, orange sooty 42, and in vial *e* a mosaic male, freak 185 (May, 1923).

Eyes and ocelli of this mosaic were mixed black and orange as shown in Figs. 1 and 2. Internal and external genitalia were normal. Mesosternum was yellow on right side, sooty on left. Sooty is very variable but asymmetry of this trait rarely occurs. Moreover, since the eyes were mosaic for orange and the mother was heterozygous for sooty, this asymmetry may be taken as representing true mosaicism. Vein r_4 was absent from the left wing, present in the right. No significance can be attached to this since the trait is commonly asymmetrical.

Freak 185 was crossed to females of orange L. stocks, 3, 5, and 10. There were produced orange males 518, of which 61 graded to sooty, and orange females, 245 or which 23 graded to sooty. These orange females indicate that the mosaic transmitted only orange.

In order to determine whether he transmitted sooty and defective, daughters were tested by breeding. From nineteen daughters of stock 3 ($od_{II95}S_{II}D_x$) females there were produced orange males, 136 of normal venation of which only 30 graded to sooty, and 160 defective of which only 28 graded to sooty. From sixty daughters of stock 5 ($oD_{II}S_{II}D_x$) females there were produced orange males, 1,926 of normal venation of which only 65 graded to sooty and 11 defective with none grading to sooty. The small proportion of F_2 males grading to sooty indicates that freak 185 lacks s_{II}. The excess of defective over normal from daughters of stock 3 females, and the presence of 11 defective from daughters of stock 5 females indicate that freak 185 transmitted d_x.

Mosaic character of eyes and mesosternum indicate that the first oöcyte division giving rise to this freak was equational for orange and probably for sooty. Defective r_4 indicates a minor factor for defect remaining in the egg. Breeding tests indicate composition of testes to be $oS_{II}D_x$. Maturation may be represented as follows:

$$\frac{\text{First polar body}\quad OoS_{II}s_{II}D_x(d_x)}{\text{Cleavage nuclei}\quad Os_{II}(D_x)\,|\,oS_{II}d_x}.$$

Freak 264.—The orange impaternate female, freak 257, $ooD_{II}D_{II}Ww$, arose from mixed ancestry and probably carried at least one minor factor for defective venation, d_x. An orange wrinkled son, $oD_{II}wd_x$, was crossed to a female of L. stock 9

($Od_{1195}WD_x$). There were produced type males 3, defective males 23, and type females 12. One of these females produced males—type 11, defective 22, wrinkled and wrinkled defective 36, orange 11, orange defective 22, orange wrinkled and orange wrinkled defective 28, and in vial *f* a male with mosaic eyes, freak 264 (June 11, 1924). The lack of females indicates that the mother had probably not mated.

Freak 264 had ocelli and left eye orange, right eye black. Figs. 7 and 8. Vein r_4 of right wing was normal, of left wing partly defective. The excess of defective over normal brothers indicates at least one minor factor for defect besides the main factor d_{1195}.

This mosaic was mated to four females of L. stock 10 ($od_{1195}WD_x$). Male offspring were as expected for stock 10, orange 36, and orange defective 252. Female offspring shown in Table I. were 309 orange and 11 black indicating mosaic character of gonads. From the last column of Table I. it may be seen that percentage of black increased between mating of June 13 A.M. and that of June 13 P.M. (difference 3.16 ± 1.10 per cent.) and again between June 13 P.M. and June 14 A.M. (difference 4.18 ± 2.08 per cent.). While these differences between successive periods are barely significant, the increase in blacks produced with an increase in age of one complete day is 7.34 ± 1.66 per cent. This may indicate that testes were of different character and that the orange-bearing was functioning sooner than the black. We were unfortunately unable to get further matings. Had this been done it is possible that the black daughters might have equaled or exceeded the orange. The black daughters differed moreover in being much more defective than orange. Blacks were 81.82 ± 7.84 per cent. defective with mean grade 2.818 while orange were 21.36 ± 1.57 per cent. defective with mean grade 0.398.

The eleven black daughters were all tested. They produced males—type 169, defective 188, orange 211, orange defective 221, and females—type 137, defective 156, orange 144, orange defective 141. They were all heterozygous ($D_{II}d_{1195}$) indicating that d_{1195} was in no case received from freak 264. Eighty-seven of the 309 orange daughters produced males—orange 1,359,

TABLE I.

DAUGHTERS OF MOSAIC-EYED MALE (FREAK 264) ARRANGED ACCORDING TO DATES OF HIS MATINGS.

Dates of Matings.	Daughters.												
	Color.	Normal.	Defective (Graded).								?	Total.	% Black.
			1	2	3	4	5	6	7	?			
June 13, A.M.	Orange	90	7	15	3	1					1	117	0.0
June 13, P.M.	Black	1	1	1								3	3.16 ± 1.21
	Orange	76	1	7	1	1				6		92	
June 14, A.M. (2 matings)	Black	1	1	2		1	1		2			8	7.34 ± 1.69
	Orange	77	6	9	5	3				1		101	
Total	Black	2	2	3		1	1		2			11	3.44 ± 0.69
	Orange	243	14	31	9	5				7		309	

orange defective 964, and females—orange 1,371, and orange defective 900. There was no correspondence in defectiveness either of black or of orange daughters of freak 264 and defectiveness of their progeny. Defective sons from black females were 51.84 ± 1.20 per cent. and from orange females 41.50 ± 0.70 per cent. The difference is 10.34 ± 1.38 in favor of the former. Defective daughters from black females were 51.38 ± 1.40 per cent. and from orange females 39.63 ± 0.69 per cent. The difference is 11.75 ± 1.56 in favor of the former.

Maturation of the egg producing freak 264 may be represented as follows:

$$\frac{\text{First polar body } \mathrm{Ood_{1195}d_{1195}wwD_xd_x}}{\text{Cleavage nuclei } \ OD_{11}Wd_x \mid oD_{11}WD_x}.$$

Freak 280.—The mutant male *reduced* (wings), freak 268, occurring in the F_2 generation from L. stock 8 female by I. stock 11 male was mated to an L. stock 1 female. A reduced male occurring in F_2 from this latter cross, mated to an L. stock 8 female produced 6 type females, no males occurring in the culture. One of these virgin females produced males—type 42, orange 39, reduced 58, orange reduced 45, and a black-eyed male, freak 280, with left primary wing long, right reduced (May, 1925).

An observed mating of this mosaic with an orange-eyed female, stock 8, resulted in orange males 98, and one black female. Unfortunately this female died without offspring and no more progeny could be obtained from the mosaic. The one black-eyed daughter demonstrates that the mosaic transmitted black.

According to the theory advanced for the production of males with mosaic eyes, freak 280 might be formed as follows:

$$\frac{\text{First polar body } \quad \mathrm{o(o)Rr}}{\text{Cleavage nuclei } \ \mathrm{O(R)} \mid \mathrm{(O)(r)}}.$$

Freak 279.—An ivory (eye) reduced female, stock 20, mated with a type male, stock 11, produced 18 ivory reduced males and 70 type females and in vial *d* a black-eyed female with left primary wing long, right reduced, freak 279 (April, 1926). This female which had evidently mated with an ivory reduced brother produced males—type 3, reduced 1, ivory 3, and females —type 8, reduced 6, ivory 7, ivory reduced 4. As far as may be judged from these small numbers, this female did not differ in her breeding behavior from her type sisters. The reduced right primary wing may be explained by some local mitotic irregularity involving a single chromosome or a reduction of the whole complex. In the latter case freak 279 may be regarded as a gynandromorph.

Gynandromorphs.

As a tentative explanation of the origin of gynandromorphs or sex mosaics in *Habrobracon* it is suggested that they may be formed in a manner similar to the mosaic males above discussed, except that one of the oötids is fertilized. There are then two cleavage nuclei, a haploid of maternal origin and a diploid of bisexual origin.

Sex of the various body regions of gynandromorphs is to be recognized by comparison with certain differences between normal males and females. Eyes show no obvious sex difference but sex of the head may be identified in almost all cases by number of joints in antennæ. The long antennæ of the male stand in contrast to the short antennæ of the female. In counts of joints in 2,407 "normal" male antennæ the modal number was twenty-three. A single antenna contained twenty-five while three had only eighteen. In counts of joints in 1,821 "normal" female antennæ the modal number was fifteen; none had over sixteen while four had as low as eleven. Deviation in "normal" individuals is therefore much greater in the minus than in the plus direction. No aberrancies have occurred increasing the number of joints but "deficiency" (Whiting, P. W., 1926*b*) reduces number in either sex. In the latter case antennal joints tend to be fused or tapering. Shortening therefore indicates antennæ as female only if joints are of normal form.

Primary wings of the female average slightly larger than of the male. Variation within each sex is so great that no reliance may be placed on this character. Obvious asymmetry in wing size may, however, be suspected to indicate sex difference between left and right sides of the same gynandromorph.

While some variation obtains in size of sternites, those of the male are much smaller than corresponding sternites of the female. External genitalia of the male bear no resemblance to sting and sensory gonapophyses of the female (Figs. 9–15).

Mating reactions of the male begin with flipping of the wings and running about excitedly when in the presence of the female or if set where a female has recently been. In small vials a male, apparently much excited, may often pass close by a female without noticing her. If, however, his antennæ come in contact with her he will mount and attempt to mate. Copulation is accompanied by a rhythmic beating of wings and antennæ. It may last from three or four seconds to two minutes and may be repeated without dismounting. A male may mate several times in close succession especially if new females are introduced. Males show much individual difference in vigor of mating reactions. The same male may show no reaction at one time and a very vigorous reaction later. Females differ in their capacity to stimulate the response. A male may be stimulated by another male and even mount him but the response is much more pronounced toward a female. A female may remain quiet and permit mating, or give a negative response by running away. If the male persists she kicks him off and bends the tip of her abdomen downward and forward in order to avoid him. He may accomplish a mating in spite of this resistance. A male which is mounted by another male acts in a way somewhat similar but since the aggressor in this case soon desists the negative response is not so pronounced. A female may rarely flip her wings a few times when first introduced to a male. It may be questioned whether this is a sex reaction or a response of a more general type.

Injury to antennæ of males interferes with normal mating reactions. If ether fumes have been too strong there may be an inability to sense the odors from the female. If contact between antennæ of the male and body of the female occurs there may nevertheless be a vigorous response. Antennæ thus injured by ether fragment after two or three days and terminal portions drop off.

Males are entirely indifferent to caterpillars, running over them hastily as over any other object such as cloth, cotton, paper, etc. Females, on the other hand, have very definite types of reactions towards caterpillars. If a female is touched by a moving caterpillar a defensive reaction occurs. She backs away with abdomen bent downward and forward and with sting extended in a threatening attitude. She then approaches cautiously and if the caterpillar moves draws back again. If the caterpillar remains active she may desist, turn about and move away. A similar threatening or defensive response may be called forth occasionally by other moving objects. If the caterpillar is quiet the female will sting it causing it to squirm violently. The wasp retreats to a safe distance until the caterpillar comes to rest. She approaches again, and again stings the caterpillar which is now unable to react, for its muscles are paralyzed. The reactions of a female wasp toward a paralyzed caterpillar consist in carefully inspecting it with the antennæ, penetrating it with the sting, and feeding from the puncture that she has made.

Oviposition normally follows some time after feeding. Ovipositing reactions begin with antennal inspection. After a favorable spot is selected, usually between the caterpillar and the glass, the abdomen is extended with sting withdrawn and eggs are laid. The wasp may remain in this ovipositing position for many minutes.

Injury to antennæ, unless very extreme, does not prevent normal reactions toward caterpillars.

Freak 247.—Along with several males and females reared at room temperature in a vial of stock 13 recently derived from crosses of L. stock 5 and I. stock there was found (January 15, 1924) an orange-eyed gynandromorph with normal wings and male head, twenty-four joints in each antenna. Abdomen was of normal female type except that first right sternite was male. Ovaries and poison sac were normal and seminal receptacle contained sperm.

Tests for reactions extended over four days. The gynandromorph kicked off males attempting to mate, bending its abdomen down slightly to avoid them. It gave positive mating response towards males, flipping wings and running after them but not mounting. Introduced to females immediately afterwards it gave aggressive mating responses,—flipping of wings, mounting, and rhythmic beating with wings and antennæ. It bent the tip of its abdomen downward in a futile endeavor to copulate. It did not thrust out its sting. Responses towards caterpillars were also like those of a typical male. Contact with an active caterpillar resulted in a simple retreat. No eggs were laid on a paralyzed caterpillar left with it for two days. Freak 247, therefore, acted in every way like a typical male.

Freak 206.—From matings between the original I. stock and L. stock 5 a certain female was isolated after having mated with her brothers. Daughters were similarly isolated in five successive generations. A few freak types appeared with extra legs, deficient digestive tract or deformed thorax. The final isolated female produced males—51 normal and 2 with antennal deficiency and females—32 normal, 1 with left mesothoracic leg and wing lacking, 1 with broadened thorax and an unexpanded wing, 1 with right eye lacking, 1 with right wing shrivelled, and 1 with abnormal arrangement of abdominal dorsites, besides a gynandromorph, freak 206, found in vial *b* (May 21, 1923).

Eyes of this gynandromorph were black like those of the parents. The specimen was hungry when found, the abdomen shrunken. It drank honey water and seemed active and vigorous. A preliminary count showed sixteen joints in one antenna which was complete, eighteen in the other which was incomplete and must therefore have had at least nineteen joints originally. Two days later it was noticed that segments were breaking off, probably due to over-etherization. The abdomen was of normal female character throughout. Ovaries, poison sac and glands were normal and seminal receptacle contained sperm.

Careful tests carried on over four days indicated no positive reactions toward females or caterpillars. To the former it appeared as indifferent as a female, to the latter as indifferent as a male. Males were kicked off when they attempted mating.

Since antennal "deficiency" occurred in this strain the shorter antenna may have been a "deficient" male antenna. There was, however, nothing abnormal in the form of the joints so that the head was probably in part male, in part female.

Freak 144.—A female of original I. stock crossed with a male from the third generation of I. by L. stock 5 produced besides 77 males and 180 females a gynandromorph, freak 144, found in vial *a* (November 29, 1922). Eyes were black as were those of its parents; head was male, twenty-two joints in each antenna; abdomen was entirely female, poison apparatus present, seminal receptacle devoid of sperm.

Freak 144 is of especial interest from the point of view of its responses. These may be described by days as the tests were made.

November 29. A.M., etherized. P.M., reacted indifferently toward caterpillars. Reacted negatively to male that tried to mate with it, turning abdomen down. Flipped wings and chased male but did not mount. Acted similarly toward female.

November 30. Showed very skillful cleaning reactions of antennæ, wings and legs. Displayed no interest in caterpillars. Flipped wings and mounted female as if to mate but soon desisted.

December 1. Virgin female introduced. Gynandromorph showed no reaction at first. Female inspected caterpillar and stuck its abdomen underneath as if to oviposit. Then gynandromorph rushed up and attempted to mate, sticking abdomen down under caterpillar near that of the female. The latter, disturbed, crawled away. Gynandromorph inspected caterpillar with its antennæ in typical female fashion for a few seconds, then left caterpillar and pursued female aggressively mounting again and again, and bending abdomen down as if to mate. Showed no more interest in caterpillar, walking over it indifferently like a male. A male, when introduced, tried to mate but the gynandromorph kicked him off vigorously, bending abdomen down and forward in typical female fashion. Gynandromorph took no interest in stung caterpillar.

December 2. Gynandromorph showed vigorous negative reaction toward males which attempted to mate. Crawled over males indifferently but flipped wings and mounted female. Showed male indifference toward crawling caterpillar with which it came into contact.

December 3. No eggs were found on caterpillar which had been left with it since November 30. Showed negative reaction as previously to male. Jabbed its protruded sting into cotton plug again and again as is sometimes done by female. Reacted toward a virgin female like a female toward a caterpillar, thrusting out sting above her and below her and showing no flipping of wings.

December 4. Negative reaction toward male. Indifferent reaction toward caterpillar.

Tests of freak 144 show clearly changes in responses between those characteristic of the male and those characteristic of the female, the male type predominating.

Freak 249.—A female from orange defective L. stock 3 was crossed with a male of orange L. stock 7. These stocks were derived early in the history of the L. material and consequently were not closely related. An F_1 female after mating with her orange defective brothers produced orange males—6 normal, 2 defective, orange females—12 normal, 8 defective, and an orange gynandromorph with normal wings, freak 249, found in vial *a* (February 8, 1924).

The head of freak 249 was obviously male, having twenty-one joints in each antenna. The abdomen was entirely female both externally and internally. Ovaries, poison sac and glands appeared normal. Seminal receptacle contained sperm, showing that a mating had taken place.

Tests for reactions extended for five days. When introduced to females, the gynandromorph responded by all the typical male reactions. A test with a male resulted in no reaction on the part of either although the male mated immediately with a female introduced shortly afterwards. Reactions toward caterpillars were altogether indifferent.

Freak 246.—After nine generations of inbreeding of descendents of the impaternate female, freak 276, two branch lines were bred for three generations separately. One gave rise to an orange-eyed female heterozygous for wrinkled wings; the other to an orange wrinkled male. A cross of these two resulted in orange males, 7 flat and 5 wrinkled; orange females, 4 flat and 6 wrinkled, and an orange wrinkled gynandromorph, freak 246, found in vial *a* (January 4, 1924).

Head was clearly male having twenty-three joints in left antenna, twenty-four in right. All structures of abdomen were female except first left sternite which was of the male type. The sting was deformed at tip. Ovaries and poison apparatus were normal, seminal receptacle devoid of sperm.

Tests for reactions extended for two days only, but were very thorough. Responses were entirely similar to those of a normal male. There was positive mating reaction toward other males but vigorous response toward females which it mounted several times. When males attempted to mate with it, it acted rather indifferent, turned abdomen down slightly and moved away. This reaction was at each time different from the definite negative reaction characteristic of certain females. No reaction toward active caterpillar could be obtained. Gynandromorph simply moved away indifferently. Even contact of caterpillar with the gonapophyses had no effect. No eggs were laid on paralyzed caterpillar left with it over night.

Freak 267.—An ivory-eyed mutant male, freak 281, appearing in F_2 from a cross of L. stock 10 female by mosaic male, freak 264, was crossed to an L. stock 10 female. Ivory defective stock 17 was derived from descendents of these. A stock 17 female was crossed to an ivory defective wrinkled male of the tenth generation descended from crosses of L. stocks 9 and 10, and descendents of the impaternate female, freak 257. An ivory defective daughter, heterozygous for wrinkled was crossed to a stock 17 male. Among the flat and wrinkled males and the flat females resulting (numbers not recorded) there appeared (February 18, 1925) an ivory defective (grade 5) flat-winged gynandromorph, freak 267.

Antennæ of this freak were definitely male although the left showed terminal fusion, deficiency, with about eighteen joints visible. The right was normal with twenty-one joints. Abdomen was entirely female externally. Ovaries and poison apparatus were normal, seminal receptacle contained sperm.

Tests for reactions extending over five days showed indifference to caterpillars. Males attempting to mate with it were repulsed many times but responses were male-like, the gynandromorph hurrying to get away rather than bending down abdomen. There was no reaction toward females until the fourth day when slight flipping occurred. On the fifth day there was aggressive reaction,—mounting, etc.

Freak 258.—An F_1 female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*) produced males—type 20, orange 12, wrinkled 15, orange wrinkled 19, and females—type 39, and a black-eyed gynandromorph, freak 258, found in vial *c* (February 26, 1924).

The head of this gynandromorph was female, fifteen joints in each antenna. Mesosternum was yellow; left wing slightly longer than right. Vein r_4 of right wing was normal, of left broken. Abdomen was entirely male with normal external genitalia. Digestive tract and abdominal nerve ganglia were apparently normal, as were the penis, sperm sac, and ducts. No testes could be found, either connected with the ducts or in dorsal anterior part of abdomen, as in the gynandromorph, freak 5.

Tests for reactions may be discussed by days abstracted from the record book.

February 27. Gave flipping reaction in presence of females. Ran over active caterpillars without noticing them. Later gave typical female reaction toward caterpillars inspecting them with antennæ, withdrawing and approaching with abdomen thrust forward.

February 28. Slight flipping in presence of various females, but in general attempted to avoid them. Definite female reaction toward caterpillars as before. Tried to sting cotton plug with tip of abdomen.

February 29. Bumped into females without flipping. Then mounted one with definite reaction as if to mate but ran off very excitedly. Inspected paralyzed caterpillar and later cotton plug very carefully with antennæ.

March 1 to March 4. Left with virgin female. No females resulted among 40 males produced.

March 4. Definite female stinging reactions towards active caterpillars. No reaction toward females. Kicked off males attempting to mate with it.

As a result of these tests we may say that reactions were predominantly female but with weak and occasional male responses.

Asymmetry of wings indicates that thorax was probably in part male, in part female. Both male and female structures therefore possess W. Female parts, the eyes, likewise have O. Since both parents possessed O and W this case gives no clue as to the origin of the characters in the gynandromorph.

Freak 250.—An F_1 female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*) produced males—type 40, orange 46, wrinkled 29, orange wrinkled 43, and females—type 62, and a black-eyed, flat-winged gynandromorph with normal r_4 vein and sooty mesosternum found in vial *c* (February 9, 1924). Head was male, antennæ each with twenty-two joints. Abdomen was entirely female. Ovaries and poison sac were normal; seminal receptacle was without sperm.

Tested with females it showed flipping of wings and mounting. It was indifferent to caterpillars but pierced cotton plug with sting. Tests extended for six days but were not entirely satisfactory as ends of antennæ broke off probably from over-etherizing.

As regards method of origin the case is inconclusive for the black eyes of the male head may have been inherited from either parent.

Freak 255.—An F_1 female, OoWw, from an orange defective wrinkled male by a sooty female, stock 11 (see *Origin of freak 250, etc.*), produced males—type 43, orange 29, wrinkled 29, orange wrinkled 25 and females—type 50, and an orange-eyed gynandromorph, freak 255, found in vial *d* (February 16, 1924).

The head of this gynandromorph was male, twenty-two joints in each antenna, wings were flat, left slightly longer than right, r_4 veins complete, mesosternum sooty. The abdomen was entirely female, ovaries and poison apparatus normal, seminal receptacle containing sperm.

No interest was shown in caterpillars. Very definite flipping reaction occurred toward females, but gynandromorph did not mount them. The tests indicate male reactions but are not satisfactory as antennæ lost their terminal joints after two days, due probably to over-etherizing.

The case is of interest from the point of view of method of origin of the male parts. Orange eyes must have come from the heterozygous mother. The flat character of the right wing, probably male, may have been received from either parent.

As regards orange, maturation and fertilization may be expressed as follows:

$$\frac{\text{First polar body} \qquad\qquad\qquad O(O)}{\text{Cleavage nuclei}\left\{\begin{array}{lr|l}\text{oötids} & o & (o)\\ \text{male pronucleus} & & O\end{array}\right.}.$$

Freak 32.—A black-eyed female from L. stock 1 was crossed to an orange male from the third segregating generation alternating with three successive crosses to L. stock 1. L. stock only was involved. There were produced black males 14 and females 20 and a black-eyed normal-winged gynandromorph, freak 32, found in vial *e* (April 20, 1921).

Head of freak 32 was male, antennæ with twenty-two joints in left, twenty-one in right. Abdomen was normal female type except that first three segments of left side were male (Figs. 22, 23, 24). Internal structures were not adequately studied. Ovaries containing large eggs were observed.

Tests extending for three days brought forth no reactions towards active or paralyzed caterpillars. Reactions toward males attempting to mate were negative, the abdomen was bent down and the gynandromorph attempted to kick off the males. Nevertheless a male succeeded in accomplishing two copulations of about thirty and fifteen seconds respectively. There was not the slightest reaction towards females. The specimen seemed healthy in every way but acted clumsily in cleaning its wings, gonapophyses, etc.

The black eyes of the male head must be of maternal origin. The mother was homozygous.

$$\frac{\text{First polar body} \qquad\qquad\qquad OO}{\text{Cleavage nuclei}\left\{\begin{array}{lr|l}\text{oötids} & O & O\\ \text{male pronucleus} & & o\end{array}\right.}.$$

Freak 248.—A black-eyed female from I. stock 11 was crossed with an orange male from L. stock 7. There resulted black males 35, black females 133 and a black-eyed, normal winged gynandromorph, freak 248, found in vial *b* (January 28, 1924).

Head was male, left antenna had twenty-two joints, right antenna was broken but there remained thirteen joints. Abdomen was entirely female, ovaries and poison apparatus were normal and there were no sperm in the seminal receptacle.

Tests extending over five days showed no reaction to caterpillars, definite negative reaction to males attempting to mate, no mating reaction toward males, but aggressive response to females, flipping, mounting, etc.

The formula for the method of origin would be similar to that for freak 32.

Freak 5.—An orange female of the third generation from the orange mutant male of L. stock was crossed with a black male of the fourth generation of Mifflintown (M.) stock. There were produced 13 orange males, 15 black females, and a black-eyed gynandromorph, freak 5, found in vial *a* (July 22, 1920).

The head of this gynandromorph was female, fifteen joints in each antenna (Fig. 17). Wings were normal. The abdomen was female in the first three segments and entire right side. The left posterior quarter was distinctly male. There was a full set of male external genitalia including penis, and first and second pairs of claspers. A portion of the sting, much malformed, and the right sensory gonapophysis were present (Figs. 18–21). Internal organs were much confused. The digestive tract appeared normal and ran dorsal to the poison apparatus which was slightly toward the right. Poison duct led from the malformed sac to the base of the sting. Poison glands were approximately normal. Seminal receptacle was normally located and devoid of sperm. Nerve ganglia were well developed. Vasa deferentia ended blindly. Testes were far out of position, lying dorsally in the anterior part of the abdomen. They were large and well-formed with cysts of spermatocytes (?) but no sperm. Ovaries were altogether absent.

Reactions of freak 5 seemed entirely female. It was indifferent to females, seemed willing to mate with males and then turned abdomen down to avoid them. It inspected caterpillars with its antennæ and tried to sting them. It also stuck its abdomen under a paralyzed caterpillar as if attempting to oviposit. The tests were all made in one day after which the specimen was killed.

Freak 5 is of interest genetically as it proves that the gynandromorph comes from a fertilized egg and that female parts show paternal influence.

First polar body		oo	
Cleavage nuclei	oötids	o	o
	sperm nucleus		O

Habrobracon brevicornis (Wesmael) which has been introduced into the United States from Europe to parasitize the corn borer may be reared on the Mediterranean flour-moth under conditions standardized for *Habrobracon juglandis*. The two species are somewhat similar. Antennæ of brevicornis are longer, normally ranging from nineteen to twenty-eight joints with a mode at twenty-five in the male, and from fifteen to nineteen joints with a mode at eighteen in the female. Sternites in the male, Fig. 32, are, as in *juglandis*, smaller than in the female, Fig. 25. Mating reactions and responses towards caterpillars are similar, but brevicornis is more excitable and active than *juglandis*. Brevicornis is likewise similar in producing males parthenogenetically. It is probable that sex determination is similar but no mutations have been obtained in *brevicornis* to check this.

Freak 269.—On May 28, 1925, Mr. A. M. Cloudman found a gynandromorph of *brevicornis* in vial *c* from a pair also producing 60 males and 46 females. The head was male with twenty-five joints in each antenna. Abdomen was apparently of normal female type, Fig. 25. Seminal receptacle was devoid of sperm, ovaries well-formed, and poison apparatus present.

In tests extending over six days no interest was shown in caterpillars, and there were no reactions towards males. The gynandromorph pierced cotton plug with its sting, bending down abdomen. There was vigorous response toward females, flipping of wings, mounting, beating of antennæ, etc.

Freak 271.—In a line of *brevicornis* bred by Mr. Cloudman a culture occurred containing a number of freak types. Besides about 75 normal males and 33 normal females, there were about 8 males with compound eyes reduced, one male with left compound eye missing and antenna twisted, 3 males with "stalked" eyes, 6 females with small eyes, and 5 dead pupæ. Moreover in vial *a* there was found (July 15, 1925) a gynandromorph, freak 271, and in vial *f* (August, 1925) 3 intersexual males (?), freaks 273, 274, and 275. Vials *f* and *g* also contained 15 males and 9 females. In freak 271 the head was male with twenty-seven joints in left antenna, twenty-six joints in right. Abdomen was female except that some of the sternites were rather small approaching the male condition, Fig. 26. Internal organs were typical female, ovaries, poison sac and glands normal and seminal receptacle devoid of sperm.

Tests extended over five days. The gynandromorph had no interest in caterpillars except that once its abdomen was curved downward toward them. Flipping reaction was shown toward males and vigorous response toward females—flipping, mounting, etc. There was also flipping reaction towards *juglandis* female.

INTERSEXUAL MALES.

Freaks 273, 274, 275.—The three intersexual males (?), sibs of freak 271, had short antennæ and male genitalia.

Freak 273, with nineteen joints in left antenna, eighteen in right, had typical male genitalia but sclerites were asymmetrical, some of them approaching the female size, Fig. 30. Freak 274 with eighteen joints in left antenna, nineteen in right, likewise had typical male genitalia and asymmetrical sclerites, Fig. 31. Freaks 273 and 274 were found dead. Their abdomens were boiled in sodium hydroxide, the chitin flattened and sternites drawn without foreshortening.

Freak 275, found alive and apparently in good condition, had eighteen joints in each antenna but the left was slightly malformed. Normal male genitalia were present and in addition a small female (?) gonapophysis, Fig. 29. Sternites were irregular, some of them approximating the female. Internal organs were typical in every way for the male.

Tests extending over seven days showed no responses to males, females, or caterpillars.

Freak 193.—A *brevicornis* female which had been exposed to her brothers produced besides 91 normal males a male (?) with an appendage similar to a small sensory gonapophysis of a female (Figs. 27 and 28). This individual, freak 193, was found in vial *b* (May 15, 1923). Sternites were similar to those of a normal male. The head was of normal male type with twenty-seven joints in left antenna, twenty-six in right. Internal organs were in every way typical for the male. No tests of responses were made.

Discussion.

Impaternate Females.

If in parthenogenesis both oöcyte divisions occur without subsequent nuclear fusion but with somatic doubling, or if the first oöcyte division is entirely reductional and the second is suppressed, homozygous individuals are to be expected.

Impaternate females of *Habrobracon juglandis*, however, may be heterozygous. Thus freak 257, homozygous for D_{II}, and, like freaks 276 and 259, for o, was at the same time heterozygous for Ww clearly demonstrating that the first maturation division must have been equational for one locus at least. Had it been reductional for Ww one of the derivatives of the first polar body must have returned to fertilize the female pronucleus in order to produce Ww. In that case, however, we would have Oo and $D_{11}d_{1195}$ unless for them the first oöcyte division had been equational.

Either one of two theories are tenable. For all three loci the first division may be equational, a derivative of the first polar body effecting fertilization or the first division may be equational for some loci, reductional for others, diploidism being effected by suppression of the second oöcyte division.

Mosaic Males.

Since mosaic-eyed male freak 185 had a virgin mother, he arose from an unfertilized egg. Since mosaic-eyed male freak 264 had no sisters, he presumably also arose from an unfertilized egg. As regards the other three males with mosaic eyes orange parts of freaks 65 and 256 and black parts of freak 154 as well as defective r_4 veins of freak 65 must have been of maternal origin.

As regards method of origin of these males it is assumed that cleavage of the second oöcyte produced a haploid mosaic (haploid mosaic arrenotoky). It is clear that reduction must have occurred as recessive characters are visible in the body and breeding tests show that gonads are haploid. In the case of freak 264, the aberrant proportion of black and orange daughters, the significant change in this proportion with advancing maturity, and the greater defectiveness of black daughters as well as their greater capacity to transmit defectiveness, indicate that gonads are mosaic rather than diheterozygous.

It may of course be assumed that any two or more of the four products of maturation took part in cleavage. If a derivative of the "first polar body" as well as the "female pronucleus" were thus involved the first division may have been either entirely reductional, entirely equational, or in part reductional, in part equational.

According to the hypothesis assumed, that the first polar body takes no part in embryo formation, the first oöcyte division must be in part reductional, in part equational.

Thus for freaks 65, 154, and 256 Oo must have undergone post-reduction; for freak 185, Oo and probably $S_{II}s_{II}$ underwent post-reduction; for freak 264, Oo and D_xd_x underwent post-reduction, while $D_{II}d_{II95}$ and Ww underwent pre-reduction. In the case of freak 280 the black-eyed male mosaic for reduced, Oo, probably underwent pre-reduction, while Rr underwent post-reduction.

Gynandromorphs.

The fourteen gynandromorphs discussed in the present paper came from bisexual fraternities. This is consistent with the theory that they arose from fertilized eggs. Freak 5, with black eyes, female head, gives critical evidence of this for its mother was orange, its father black. It also precludes the possibility of matroclinous female parts developed thelytokously. Three parents of the two gynandromorphs of *brevicornis* and of five of *juglandis* had no obvious factorial differences other than sex. The character shown by three others was possessed by both parents, the mothers being heterozygous.

Freaks 32 and 248 with male head, black eyes, had black mother and orange father, while freak 255 with male head, orange eyes, had heterozygous mother, black father. Male parts of these three must therefore have been derived from the mother. In the case of freak 255 at least reduction occurred.

The evidence does not prove although it is consistent with the theory that male parts are from one oötid, female parts from a fusion of another oötid and the male pronucleus.

Intersexes.

The fundamental distinction between gynandromorphs and intersexes is that the former are genetic mosaics while in the latter all parts of the body are presumed to be of similar genetic constitution. Male and female parts of gynandromorphs occur in distinct regions while intersexes are either male with a greater or less female tendency or female with a greater or less male tendency.

Intersex, freak 193, occurred in a male brood, hence in all probability from an unfertilized egg. It is therefore to be considered as an intersexual male.

Intersexes, freaks 273, 274, 275, occurring in one culture, differ from a gynandromorph in that the male and female regions are not definitely defined. They are considered to be male intersexes although their short antennæ may indicate that they are gynandromorphs with female heads.

Freak 271, a sib to freaks 273, 274, and 275, with male head, male responses and female abdomen should be regarded as a gynandromorph. Its irregular sternites and relationship to intersexual males may indicate that it is also intersexual.

Behavior of Gynandromorphs.

Responses of mosaic males and of impaternate females do not differ from responses of normal males and females respectively.

The fourteen gynandromorphs and one intersex of *Habrobracon* tested may be grouped with respect to responses as follows:

(1) Typically male. *Juglandis* freaks 246, 247, 248, 249, and 267 were definitely and strongly male. *Juglandis* freak 255 was definitely male but failed to mount females. This failure may be due to injury of antennæ by ether. *Juglandis* freak 250 and brevicornis freak 269 were strongly male but each of these was seen to pierce the cotton plug with its sting, a reaction characteristic of females. The meaning of this reaction is not clear as it is not known what males would do if they had stings. *Brevi-*

MARINE BIOLOGICAL LABORATORY LIBRARY WOODS HOLE MASS.

cornis freak 271 was definitely and strongly male, although it was once observed to direct its sting toward a caterpillar. These nine gynandromorphs had long male antennæ. Abdomens were typically female except for one anterior sternite in 246 and 247, and some irregular reduction of anterior sternites in 271 (Fig. 26).

(2) Predominantly male. *Juglandis* freak 144, although showing definite and vigorous responses toward females, and for the most part no interest in caterpillars, had a definite but very brief female response toward the latter. Moreover it showed decided negative reaction toward males attempting to mate with it and inspected and pierced the cotton plug in a way characteristic of females. It had male antennæ and female abdomen.

(3) Predominantly female. *Juglandis* freak 258 was predominantly female, reacting toward caterpillars and piercing cotton plug. It seemed in general less interested in caterpillars than a normal female and frequently gave flipping reactions toward females, at one time mounting and attempting to mate. It had female antennæ, male abdomen but no testes.

(4) Typically female. *Juglandis* freak 5 acted in every way like a typical female. It had a female head, but a mixed abdomen. Lack of ovaries and presence of poison apparatus and testes are to be noted.

(5) Indifferent. Complete indifference of *juglandis* freak 206 may possibly be ascribed to overdose of ether but this is unlikely. Its female abdomen, one long and one short antenna, might be supposed to cause conflict of tendencies resulting in failure of response. *Juglandis* freak 32 was perfectly healthy but completely indifferent. It had head and first three left abdominal sternites male (Figs. 22, 23, 24). It is possible that had it been kept alive longer than three days it might have reacted toward females, although others gave aggressive responses when much younger. The indifference of *brevicornis* freak 275, intersexual male with short antennæ, mixed abdomen and normal male internal organs, cannot now be explained.

Disregarding the indifferent groups (5) as being not satisfactorily explained as yet we may say that responses are controlled by the head but that there are conflicting tendencies in certain instances.

Presence of ovaries in group 1 and group 2, lack of gonads in group 3, and presence of testes in group 4, show failure of gonads to determine type of response.

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EXPLANATION OF PLATE I.

FIGS. 9–24. Camera lucida drawings of various parts of gynandromorphs of *Habrobracon juglandis*, freaks 5 and 32, and of normal males and females for comparison.

FIGS. 9, 10, 11. Dorsal, sinistral, and ventral view of tip of abdomen of normal male. × 50.

FIGS. 12, 13, 14. Postero-sinistral, sinistral, postero-ventral views of tip of abdomen of normal female. × 50.

FIG. 15. Posterior view of tip of abdomen of normal male. × 50.

FIG. 16. Sinistral view of abdomen of normal male. × 22.

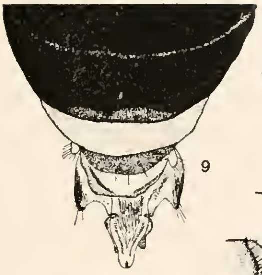
FIG. 17. Anterior view of head of freak 5. × 32.

FIGS. 18 AND 19. Postero-sinistral and sinistral views of tip of abdomen of freak 5. × 50.

FIGS. 20 AND 21. Dextral and ventro-sinistral views of abdomen of freak 5. × 32.

FIG. 22. Dextral view of freak 32. × 9.

FIGS. 23 AND 24. Sinistral and ventral views of abdomen of freak 32. × 17.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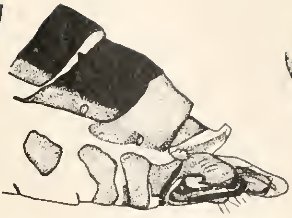
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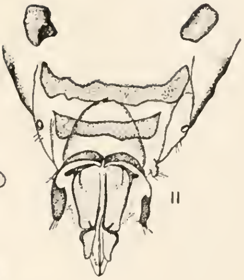
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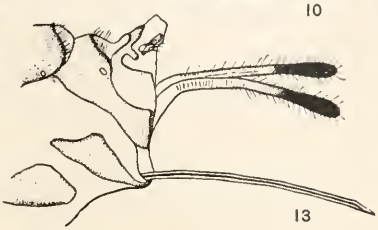
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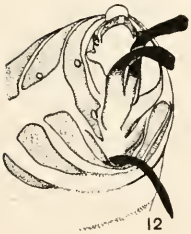
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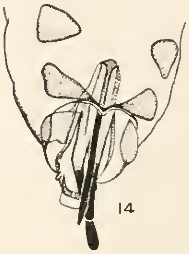
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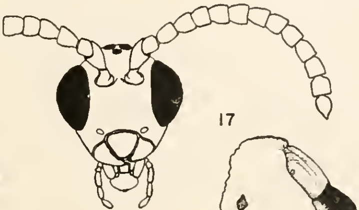
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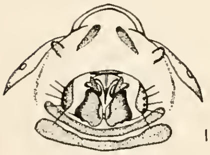
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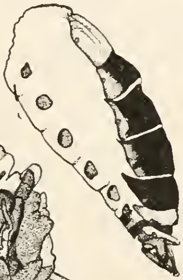
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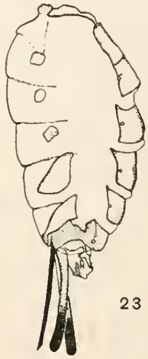
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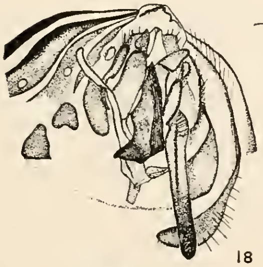
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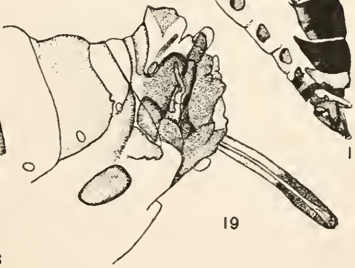
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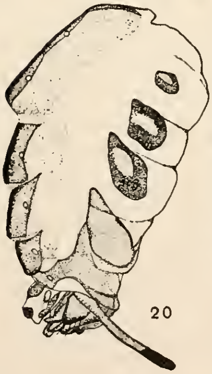
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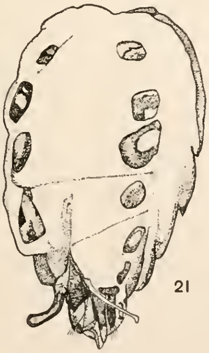
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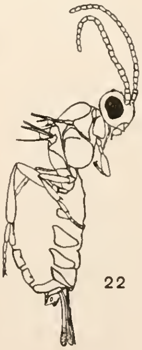
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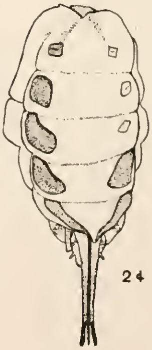
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P. W. WHITING AND ANNA R. WHITING.

Explanation of Plate II.

Figs. 25–32. Abdominal sternites and external genitalia of gynandromorphs and intersexes (?) of *Habrobracon brevicornis.* × 29.

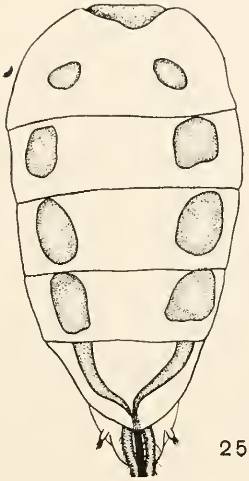
Figs. 25 and 26. Ventral views of abdomens of gynandromorphs, freaks 269 and 271. Fig. 25 is typical for the female.

Figs. 27 and 28. Ventral view of abdomen and dextral view of tip of abdomen of intersexual male (?), freak 193.

Fig. 29. Ventral view of abdomen of intersexual male (?), freak 275.

Figs. 30 and 31. Abdominal sternites of intersexual males (?), freaks 273 and 274.

Fig. 32. Abdominal sternites of normal male.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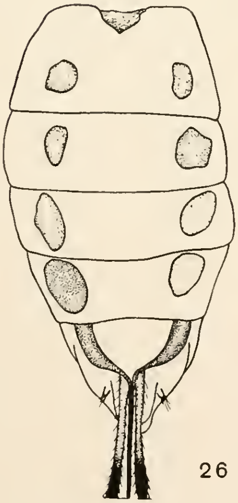
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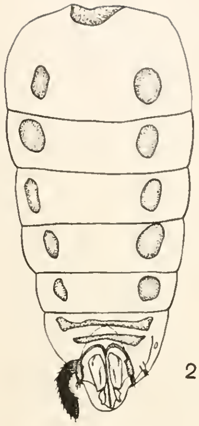
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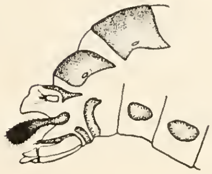
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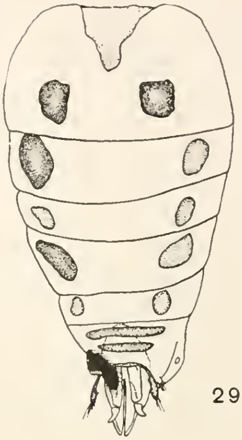
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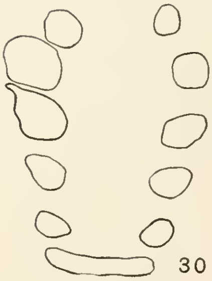
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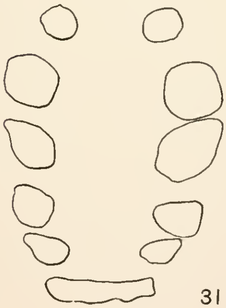
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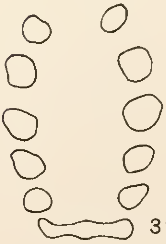
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P. W. WHITING AND ANNA R. WHITING.

SEX-INTERGRADES IN FŒTAL PIGS.

(Preliminary Report.)

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The hormone theory as an explanation of the type of sex-intergrade known as the free-martin has hitherto been limited in its application to conditions in cattle. The theory permits however, general applicability, and all species that are able to duplicate the conditions which in bovine species invariably produce the free-martin, would be expected to give rise to similar anomalous forms (Lillie, '17).

The conditions essential for the production of the free-martin are the establishment of a common circulation between a heterosexual pair of individuals at an early stage in their embryonic development. Thus certain sex-hormones liberated into the embryonic blood stream are common to both individuals, and succeed in modifying the normal course of sexual growth. The fact that it is invariably the female structures which are affected, and that they are modified in the male direction, leads to the further assumption that the male hormones are more precocious in their development than those of the female, and consequently are able to exert their influence without any counteraction on the part of the female. This assumption receives considerable support from histological evidence in that it has been observed that the interstitial cells, the supposed seat of sex-hormones, develop earlier in the testis than in the ovary. In cattle, such cells first occur in testes of 3 cm. embryos, and in ovaries of 82 cm. females (Bascom, '23). A similar precocity of male interstitial cells has been stated to occur in the pig (Allen, '03).

Species which are typically uniparous and in which the placenta is of the diffuse type, offer the most favorable opportunities for the production of chorionic fusions and resulting complications.

In the pig, the two apices of the chorionic vesicle do not enter into formation of the placenta. The chorion is thin in these regions, being stretched by the extension of the allantois inside. These terminal regions are practically devoid of blood vessels, and remain throughout the whole period of gestation as thin necrotic areas which may closely adhere to, or become invaginated into similar regions of adjacent vesicles. These, in virtue of their necrotic structure, prevent actual fusions of chorionic tissues.

Chorionic fusions may be occasionally found in swine, however. Blastocysts may come to lie either side by side or end to end, and then fuse. In each case obliteration of the intervening wall results in the formation of a single chorionic cavity. Such fusions are not often followed by vascular anastomoses, for the vessels of the individual circulations do not often come into close proximity. They may frequently be seen completely reversing their direction upon approaching each other. The total free-martin conditions are consequently of comparatively rare occurrence in the pig.

Investigation into this situation was prompted by the discovery of a pair of heterosexual pig twins among some twinning material kindly supplied by Dr. Newman, University of Chicago.

I would like to express my thanks to Dr. Newman for kindly placing his material at my disposal, and also for helpful advice throughout this work. To Dr. R. F. Shaner, University of Alberta, I am also indebted for valuable criticism, and to Swift and Co. for coöperation during the collecting of material used for this work.

An examination this summer of approximately 400 uteri in the stage of mid-pregnancy yielded 7 cases of twinning. These, with 6 collected the previous summer, and 4 from the collection of Dr. Newman, make a total of 17, of which 8 are judged to be dizygotic in origin, 3 monozygotic, 4 undetermined, a pair of conjoined twins and a double-headed monster.

Seven instances of inter-embryonic vascular connections were observed. In most of these, the anastomoses were of side branches and therefore comparatively weak. In two cases the twins showed a marked difference in size, accompanied by injuries of the smaller individual. There was here, I take it, an unequal sharing of the blood supply, one twin receiving an excess of blood and thereby robbing its co-twin of the full amount required for normal development. In one instance, the twins measured 30 mm. and 13 mm. respectively. The smaller individual had not commenced disintegration, but was a case of acardia with several morphological abnormalities.

In 4 cases, the establishment of vascular inter-communications was followed by abnormalities of the sex-equipment of one of the component twins. I now propose to describe these four cases.

Case No. 1.—(Figs. 1 and 3.) The twins in this case measured 65 mm. and were heterosexual as determined by the external genitalia. The normal length of the chorionic vesicle, and the position of the embryos in its center, indicate that fusion had taken place in a side-by-side manner. Fig. 1 is a semi-diagrammatic representation of the chorionic vesicle and its contents. In the foreground is the amnion enclosing the female embryo (*A*), behind which its allantois extends the complete length of the exocœlom. The constriction in the allantois to the right of the embryo is no doubt caused by pressure of the male embryo (*B*), which lies behind allantois *A* at this point. The allantois of *B* lies in the background, and also extends the complete length of the vesicle.

The distribution of the allantoic vessels is irregular. The greater portion of the placenta is vascularized by embryo *A*, whose anterior and posterior paired vessels are of equal strength and course the total length of the chorion. Those of *B* are unequal, the anterior pair being extremely weak and lost to view upon entering the placenta. The stronger posterior pair courses side by side with the corresponding pair from *A*, and in more than one place the branches of the two systems anastomose.

On dissection of the embryos, the reduced size of the female gonads was at once apparent. Actual measurements were 2.2 mm. in length compared with 4.4 mm. of the testes. A difference as great as this I have not observed in any normal individuals at the same stage of development. Histological examination showed structural modifications similar in most respects to those described for early free-martins. Figs. 5, 6 and 7 are camera lucida drawings through the widest parts of gonads of the female twin, a normal female, and the male twin. The reduced size of the free-martin ovary is at once apparent, such inhibition of growth being, as in bovine forms, the initial stage of modifications brought about by the action of the male hormone. In addition to reduction in size, there is also inhibition of characteristic ovarian differentiation. In the male, the germinal epithelium is reduced to a single layer of cells, and is separated from the primary sex cords by a well-differentiated layer of fibrous connective tissue. The rete occupies a central position, and radiating from it are the primary sex cords. In the normal female, the germinal epithelium has undergone extensive proliferation, and now forms a thick cortical zone of secondary cords. The primary albuginea lying between the medullary and the secondary cords is embryonic in type, and lacks the character of the male structure. The medullary portion of the ovary is occupied by the rete, and a few remaining medullary cords. In the free-martin gonad the cortical zone of secondary cords is practically absent. These cords commence their proliferation from the germinal epithelium in 25 mm. embryos (Allen, '03). Consequently their absence in a 65 mm. individual indicates decided inhibition of female differentiation. The rete is conspicuously large, but most of the medullary cords have undergone degeneration, and are present as an unorganized mass of cells. The cortical region consists largely of connective tissue, not so well differentiated as in the male, but forming, nevertheless, a definite layer beneath the epithelium. The germinal epithelium has for the most part been reduced to a single layer of cells, but in a few places there is what I take to be the initial stages of sex cord formation. A few clumps of cells appear to have been cut off from the epithelium, and are wandering in the albuginea where they are apparently undergoing degeneration and absorption.

Differentiation of the genital ducts has barely commenced in embryos of this age, therefore irregularities have not occurred to any great extent. The narrowing of the Wolffian duct which takes place in normal females of this age, had not occurred in the free-martin.

Case No. 2.—This case is practically a repetition of the one just described. The twins measured 45 mm. and were heterosexual. Their position in the chorionic vesicle and the orientation of their membranes was similar to case No. 1. Anastomoses of veins occurred in places marked in Fig. 4. The same abnormality of the female sex-equipment was present here also, but this being a slightly younger stage, the medullary cords were more conspicuous, and still retained their cord-like structure. The cortical zone of secondary cords was again represented by a few clumps of degenerating cells in the tunica albuginea.

Case No. 3.—(Fig. 2.) This case differed from the previous in that the monochorionic condition had arisen from end-to-end fusion of blastocysts. This was evident both from the double length of the chorionic vesicle, and from the orientation of the embryos and their membranes. The zone at which fusion had occurred was indicated externally by a mere thinning of the placenta which stretched uninterruptedly across this zone, without leaving any signs of imperfect fusion in the form of persistent necrotic areas. The blood vessels of the two circulations here came into contact and inter-communicated by means of distinct venous anastomoses.

The fœtuses measured 9 cm. Modifications of the female sex-equipment have resulted in small testis-like gonads completely devoid of any trace of secondary sex cords (Fig. 8). In no place is the germinal epithelium more than one cell in thickness, indicating that secondary sex-cords, if present earlier, have by now been completely re-absorbed. The underlying tunica albuginea is typically male in structure, even to the extent of penetration of blood vessels. The medullary region is conspicuous and contains numerous sex cells arranged in clumps. These are being surrounded by connective tissue growing in from the tunica, and have thereby the appearance of early seminiferous tubules.

The Wolffian ducts were large throughout their entire course, not showing the normal degeneration at their anterior portions, and the Müllerian ducts had followed the male course of growth in becoming discontinuous in the region immediately posterior to the gonads.

Case No. 4.—(Figs. 11 and 12.) This case presents altogether a different condition from the three previous. A free-martin is a zygotic female co-twin to a male. The twins in this case were two males and measured 6.3 cm. and 7.8 cm. respectively. Their position and orientation in the chorionic vesicle was identical with that of cases Nos. 1 and 2. The vascular anastomosis was stronger than in any other case and was doubtlessly responsible for the difference in rate of growth of the two embryos. Consequent upon an excess of the blood supply being received by one twin, the less fortunate individual was deprived of the full amount necessary for complete growth and normal development.

The external genitalia of both individuals was typically male in structure. The scrotal sacs, which were plainly marked in the larger male, however, were indiscernible in the smaller.

Examination of the internal genital systems, showed the larger individual to be quite normal. The testes, measuring 4.4 mm. by 2.2 mm. had not commenced their descent. The mesonephroi were large and without any signs of shrinkage. Fig. 9, representing a section through the urogenital folds immediately posterior to their fusion, shows the large size of the Wolffian ducts. The Müllerian ducts persist in this region, but farther anteriorly become intermittent.

The smaller individual presented the condition illustrated in Fig. 12. The testes measured 3.9 mm. in length and 1.2 mm. in width. Descent was in progress, the left having out-distanced the right by more than its own length. Degeneration of the mesonephroi which occurs normally in pigs of 10 cm. or more, is here precocious and irregular. All that is left of the left mesonephros is a narrow ridge of tissue accompanying the genital ducts. The right has retained its normal size at its anterior end and is extremely irregular in its posterior region.

On section, the testes proved to be normal in structure. Compared with those of the co-twin, they were more primitive however, being only one half the diameter, and possessing a fewer number of tubules. The tubules contained fewer sex-cells, and a great abundance of inter-tubular material was present.

Fig. 10, representing a section through the genital ducts at a region corresponding to that in Fig. 9, indicates the difference in size of the two Wolffian ducts. The left duct is approximately as small as its accompanying Müllerian duct, and even that on the right does not approach the normal size. The Müllerian ducts could be traced throughout their whole length, and the left was especially large. Degeneration does not occur in the region indicated in diagram until a later date, hence no irregularities in size are evident here.

Discussion.

A considerable number of sex-intergrades in adult swine have been described during the last few years. The free-martin condition as a possible explanation has been discussed and abandoned partly on the grounds that chorionic fusions with their complications have not been described in the pig. There is no doubt that the first three cases described in this paper would have developed into typical sterile free-martins such as occur in cattle. Their gonads might be structural testes, or, in consideration of the fact that a certain amount of secondary sex-cord proliferation takes place, they might possibly be ovo-testes. The temporary appearance of the Pflüger cords and their consequent degeneration, as described in the first two cases, is coincident with the development of the interstitial cells in the female gonad. It has been pointed out by Allen ('03), that these cells first appear in scanty quantities in the ovary, at the same time as they appear in the testis in large numbers, but that they subsequently disappear in the ovary not to reappear until a much later period of development. With their disappearance the male hormone is able to exert its influence without any counteraction on the part of the female, and degeneration of the few female sex-cords results.

Case No. 4 presents a different proposition. Similar abnormalities in adult swine have been explained by Crew ('23) on the hypothesis of quantitative sex-differentiating stimulus. A minimum amount of stimulus is required at certain critical moments during development for complete organization of one sex and suppression of the other. A retardation in the production of such stimulus, or an insufficiency in quantity thereof, results in incomplete organization of the one sex and suppression of the other. In the case under discussion, insufficiency of the required stimulus may be supposed to have been caused by the loss of blood following the establishment of a common circulation between the twins. This took place after differentiation of the external genitalia, and consequently these are normal in structure. Later developments, including complete differentiation of the genital ducts, degeneration of the mesonephroi, growth and descent of the testes, have been affected, and these therefore show evidence of irregular and uncontrolled growth.

These are only suggestions, however. More material is required to clear up these doubtful points, and it is hoped that further investigation will determine more definitely the extent to which intra-uterine conditions are responsible for the production of sexual abnormalities in the pig.

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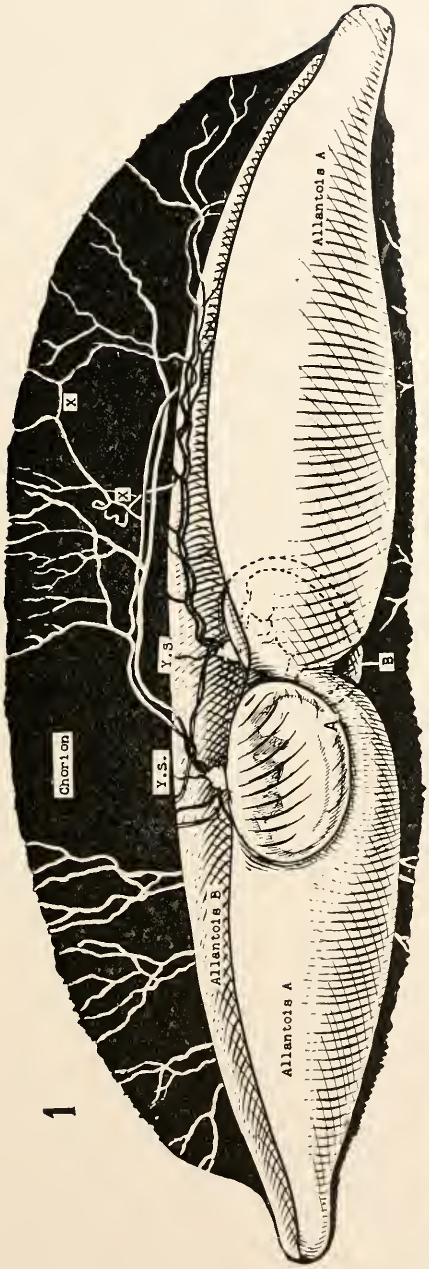
Plate I.

Fig. 1. Case No. 1. *A.*, amnion enclosing female twin; *B.*, amnion enclosing male twin; *Y.S.*, yolk-sac; *X.*, anastomoses of vessels.

Fig. 2. Case No. 3. Terms as in preceding figure.



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Chorion
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X
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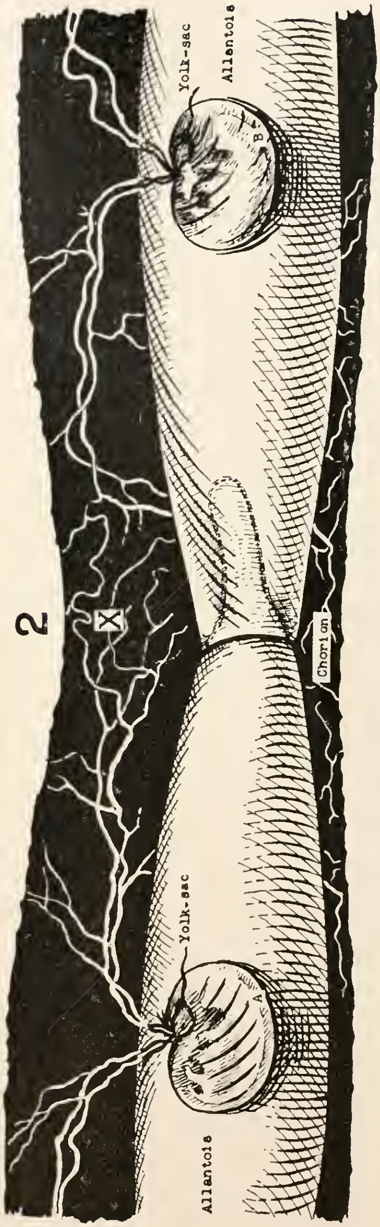
Y.S.
Allantois B
Allantois A
Allantois A
A
B



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Yolk-sac
Allantois
B
X
Chorion
Yolk-sac
A
Allantois

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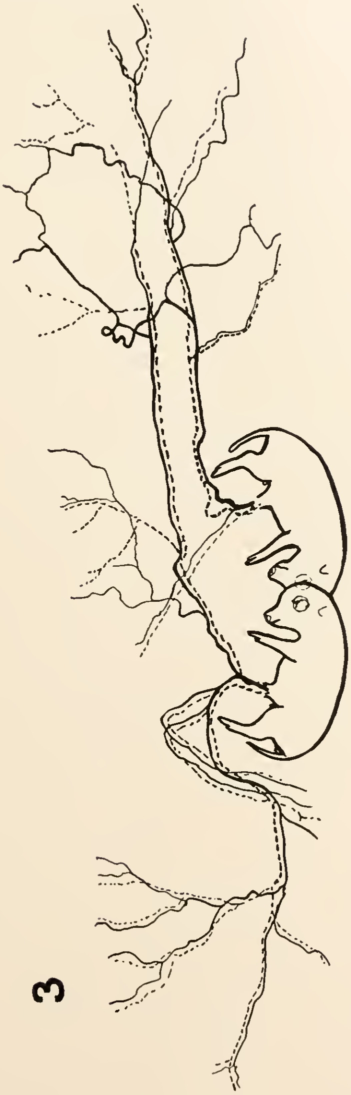
PLATE II.

FIG. 3. Case No. 1. Embryonic membranes removed to show vascular systems.

FIG. 4. Case No. 2.



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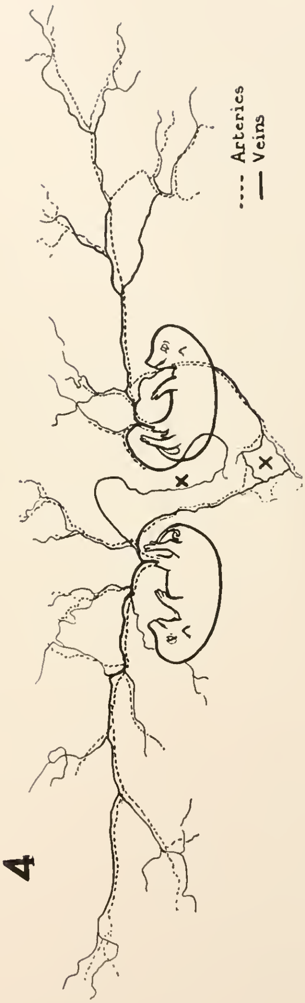
---- Arteries
— Veins

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WINIFRED HUGHES.

PLATE III.

FIG. 5. Modified ovary of free-martin, case No. 1.

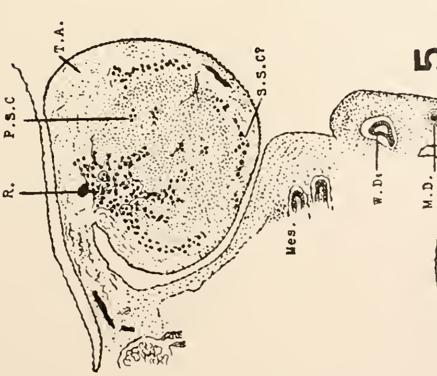
FIG. 6. Ovary of normal 65 mm. embryo.

FIG. 7. Testis of male twin case No. 1.

FIG. 8. Modified ovary of free-martin No. 3.

FIG. 9. Genital ducts of normal male twin, case No. 4.

FIG. 10. Genital ducts of abnormal male twin, case No. 4. *Mes.*, mesonephros; *M.D.*, mullerian duct; *P.S.C.*, primary sex cords; *R.*, rete; *R.O.*, rete ovarii; *R.T.*, rete testis; *S.S.C.*, secondary sex cords; *T.A.*, tunica albuginea; *W.D.*, wolffian duct.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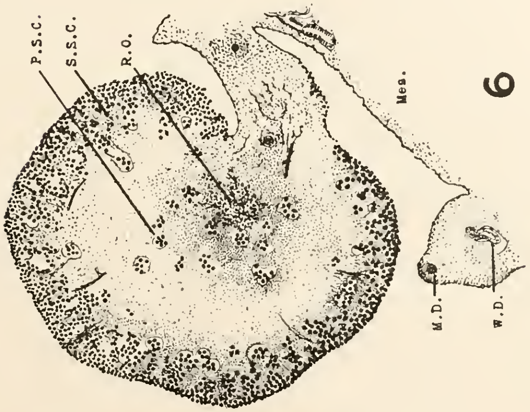
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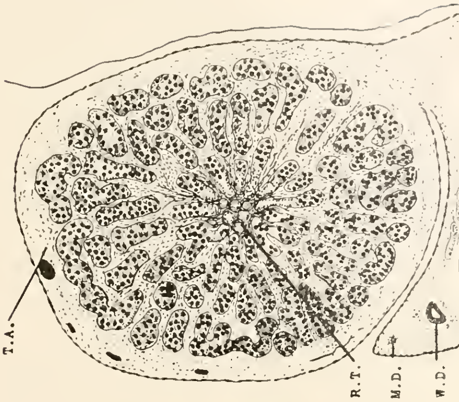
T.A.
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W.D.
Mes.

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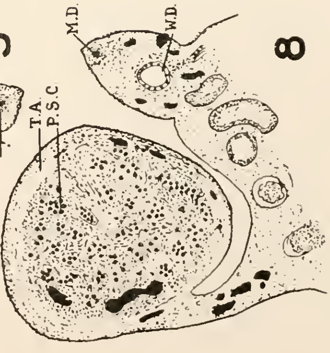

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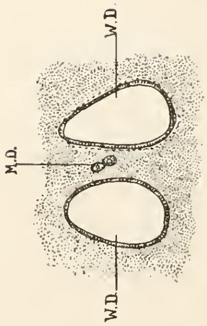

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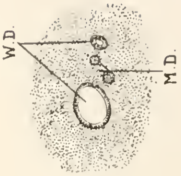

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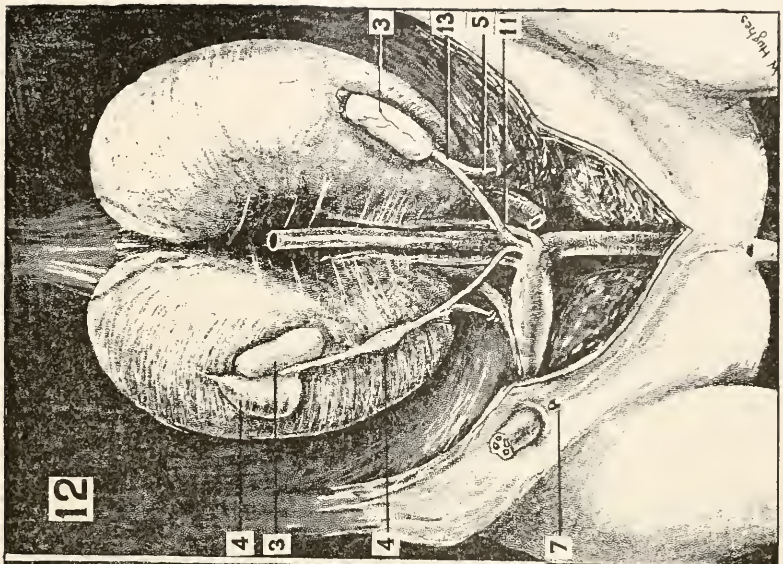
WINIFRED HUGHES.

Plate IV.

Figs. 11 and 12. Normal and abnormal twins of case No. 4. 1, kidney; 2, gut; 3, testis; 4, mesonephros; 5, inguinal fold; 6, umbilical cord; 7, penis; 8, allantois; 9, prostatic urethra; 10, scrotal sacs; 11, ureter; 12, allantoic artery; 13, genital ducts.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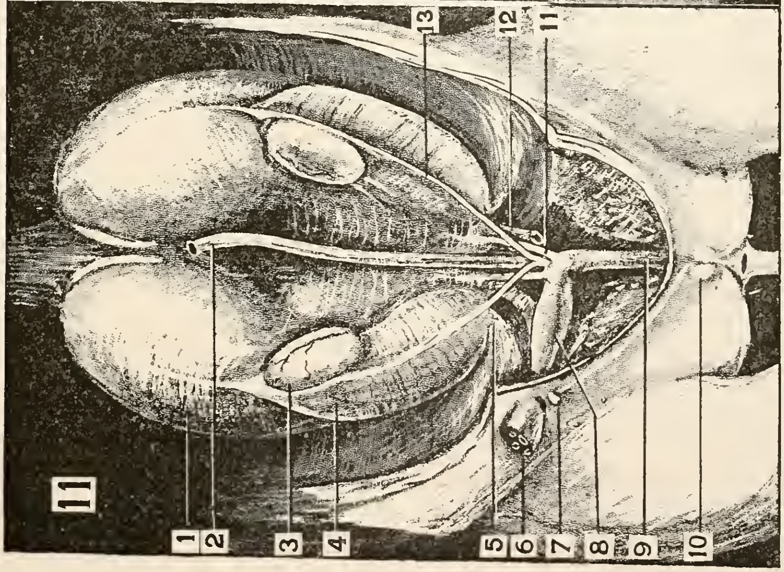
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WINIFRED HUGHES

SEX-REVERSAL IN PARABIOTIC TWINS OF THE AMERICAN WOOD-FROG.[1]

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On the basis of his work on the free-martin F. R. Lillie (1917) expressed the view, "that sex-determination in mammals is not irreversible predestination, and that with known methods and principles of physiology we can investigate the possible range of reversibility." Numerous investigators followed this suggestion in an effort to determine the nature of the sex-differentiating factors.

Parabiosis experiments which were first performed in rats by Morpurgo in 1908, were carried out by Matsuyama (1919), Yatsu (1921) and Pfeiffer and Zacherl (1926). These authors obtained almost identical results in so far as there was no sex-change observed. Parabiosis here does not even prevent copulation or suppress fertility in either sex. Yatsu describes some pathological changes taking place in the ovaries and the uteri of the female, while the male co-twin is never impaired in the least by the female. But as the castrated male exerts the same influence as the normal one, it seems improbable, that we have to do in this case with a specific action of male differentiating factors.

The experiments were then extended upon birds and amphibians, in which the embryos are accessible for the experiment long before their sexual differentiation. Minoura (1921) first reported some intersexual features in the female chick embryos, caused by implanted pieces of the testes of adult cocks. But Greenwood (1925), when repeating that experiment did not find such influences. Recently Willier (1925) grafted embryonic chick gonads, shortly before or after their sexual differentiation, into the chorio-allantoic membrane of nine-day-old host embryos. The material was preserved nine days after the operation. In no case of heterosexual combinations was there found a specific modification in the process of sex-differentiation of the graft, nor any trace of sex-reversal in the gonads of the host embryo.

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[1] The experiments were carried out in the Osborn Zoölogical Laboratory, Yale University. The microscopical study of the material was continued at the Marine Biological Laboratory, Woods Hole, Mass. The writer takes this opportunity of expressing his obligation to these institutions and feels also very much indebted to the International Education Board for the grant of a fellowship.

Briefly, we can say that the experiments with mammals and birds failed to show a definite indication of a tendency towards a physiological sex-reversal.

On the other hand Burns (1925) reported a very successful experiment on the urodele *Amblystoma*. He joined young embryos during the tail-bud stage in parabiosis. Instead of the expected chance combinations of the sexes, requiring a ratio of 1 ♂♂ : 1 ♂♀ : 1 ♀♂ : 1 ♀♀, he obtained exclusively one-sexed pairs.[1] Burns is inclined to interpret the numerical result of 44 ♂♂ + 36 ♀♀ as a 1 : 1 ratio and infers, that in about one half of the original ♂♀ and ♀♂ combinations the males changed into females, while in the other half, the females changed into males. He claims, that "there is no prepotency, which constantly favors a given sex." But he did not happen to see any developmental stage of the supposed sex-transformation. He finds the pairs to be one-sexed already in the very earliest phases of sex-differentiation. Therefore the essential part of the process remains obscure. Burns' experiments, although successful in showing that a transformation of sex occurs, did not solve the problem of how it occurs.

In the spring of 1926 the writer started new experiments in which he joined young frog embryos in parabiosis. The operation was performed shortly after the closure of the medullary tube; that is, at an age of approximately 50 to 70 hours, when the eggs were kept in the laboratory at room temperature (18°–20° C.). In normal controls the first sex-differentiation takes place during the third week. In the parabiotic twins it is often somewhat delayed. The twins were preserved at intervals during the larval period and the stage of metamorphosis (Fig. 1).

The material belonged to four different species, but in the following we consider only the experiments with *Rana sylvatica*, the American wood-frog.

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[1] ♂♀ means male on the left, female of the right side; and vice versa.

Controls were reared under identical laboratory conditions. Sex-differentiation was in full course by the 22d day, in a lot of larvæ, preserved at this time. The animals metamorphosed between the 44th and the 59th day. The species proved to belong to the differentiated sex type. Among these 196 controls were 100 females and 96 males—a very close approximation to a 1 : 1 ratio (expectation calling for 98 : 98 with a probable error of ± 4.7).

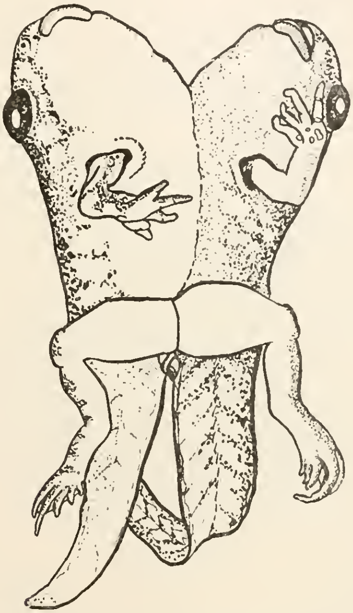

FIG. 1. Parabiotic twins TS1. Newly metamorphosed. The left side animal with the pigmented ventral surface is an European *Temporaria*, the right side animal is an American *Sylvatica*.

We should therefore expect, that by chance combination, the 56 twins considered in this paper would consist of the following zygotic groups—14♂♂ + 14♂♀ + 14♀♂ + 14♀♀ (the probable error being ± 2.2). Preliminary observations on a part of this material seemed to indicate that in the heterogeneous combinations the sex of one animal is not influenced by that of the other, and a statement to this effect was made in an evening lecture at the Woods Hole Laboratory this summer.

There are indeed a considerable number of cases like No. 44, in which one animal possesses normal testes, while the other has ovaries, which do not show any apparent deviation from the typical female conditions (compare Figs. 2 and 3). Later investigations, however, showed, that some of the females twinned with males underwent sex-reversal. In three such cases large parts of the gonads show a nearly typical testicular structure (Fig. 4), while other parts are ovarial or show progressive stages in the transformation process. In seven more cases the gonads are in the first stages of that transformation (Fig. 5). Such ovaries are, without exception, very slender. The same thing is observed in some cases, in which the reorganization has not yet begun. Obviously an *inhibition in ovarial growth* is the first visible effect exerted by the male co-twin.

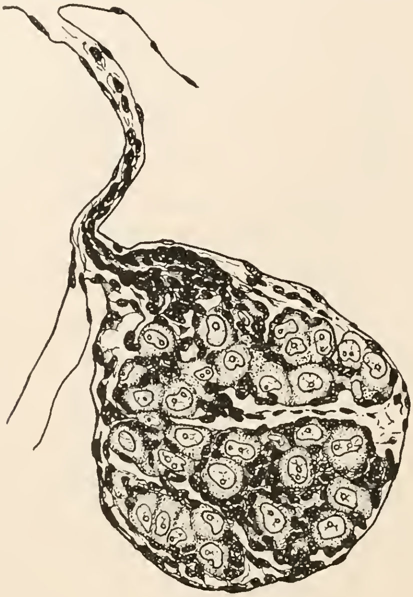

FIG. 2. Parabiotic twins SS44. (Both are *Rana sylvatica*.) Left larva, 34 mm. total length. 68 days after the operation. Male. Transverse section through one of the testes. The germ cells (primary spermatogonia) are in the sex cords. × 444.

From the fact, that the reverse combination of a female with a sex-changing co-twin was never found, it becomes evident, that *the male sex dominates the female one.*

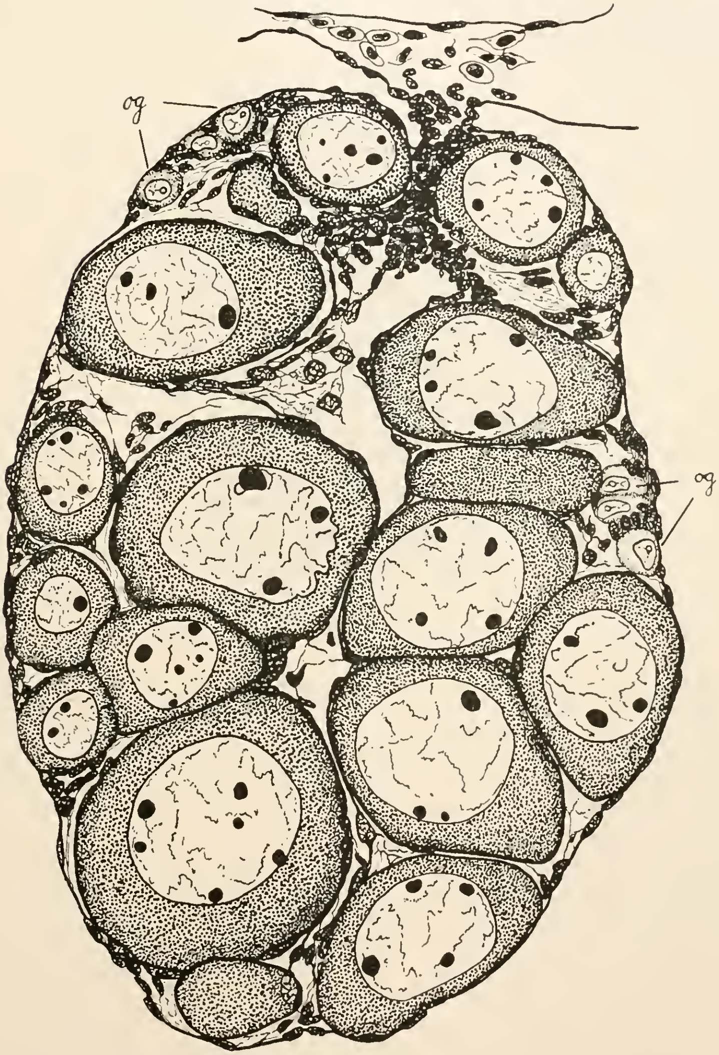

FIG. 3. Same pair SS44. Right larva, same length as left one. Female. Transverse section through one of the ovaries. The germ cells (primary oögonia and auxocytes) are in the peripheral germinal epithelium. *og.*, primary oögonia. × 444.

With regard to the last two points, the above statements are in full agreement with Lillie's observations on free-martins. However, while the latter never effect a complete sex-change, and do not produce male germ cells, the changing frog twins apparently are on the way to become true males. In frogs, exhibiting a transformation of sex, the undifferentiated germ-cells, which in the ovary are exclusively found in the peripheral germinal epithelium (so-called primary oögonia, Fig. 3, *og.*) migrate to the center of the gonad (Fig. 5, *mig.*). They join the sex-cords, to form the characteristic lobules, which are known to be the anlagen of the seminiferous tubules (Fig. 4), (Witschi, 1914; Swingle, 1926). From this stage they are therefore to be considered as *spermatogonia* (Fig. 4, *spg.*).

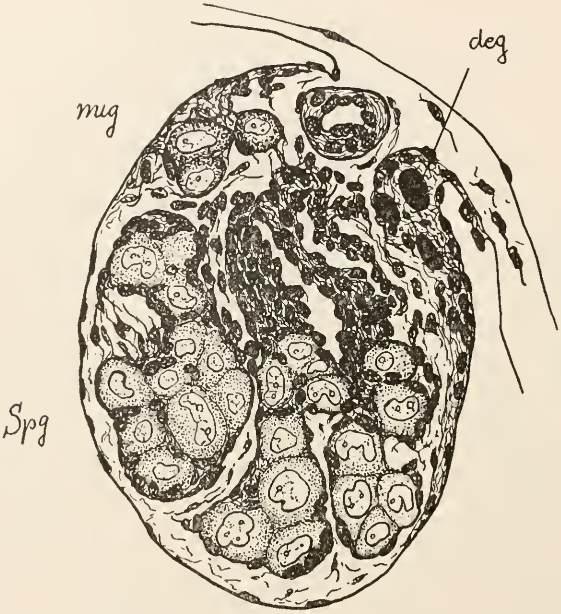

FIG. 4. Parabiotic twins SS40 (*R. sylvatica*). Right animal shortly after metamorphosis. 68 days after the operation. Female changing into male. Transverse section through the posterior part of the gonad which shows nearly typical male conditions. Highly developed sex cords, relatively small number of spermatogonia. Rests of the former germinal epithelium at *mig.* (migrating germ cells) and *deg.* (degenerating oögonia). × 444.

The described facts are in many respect in apparent contrast to those reported by Burns (1925) in *Amblystoma*. This author did not find that the male sex predominates or even that sex-transformation decreases the growth of the involved gonads. Furthermore, he supposes that the change of sex takes place before the time of sex-differentiation. Let us consider the latter point with respect of the present experiments with *R. sylvatica*.

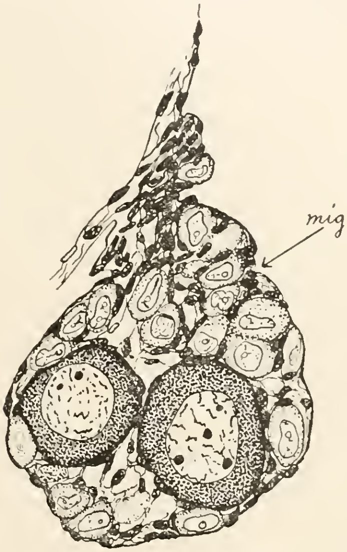

FIG. 5. Parabiotic twins SS41 (*R. sylvatica*). Left larva, 38 mm. total length. 68 days after operation. Female changing into male. Transverse section through one of the gonads, showing inhibition of growth, disintegration of the germinal epithelium and formation of the testis in the center of the gonad. *mig.*, germ cells migrating into the sex cords. × 444.

Supposing there were such an early sex-reversal in the frog twins, then the number of one-sexed pairs ought to be increased at the expense of the two-sexed ones. However, the statistical evidence is against such an assumption. There were found the following combinations:

♂ ♂—16
♂ ♀—17 (seven of the females undergoing sex-reversal)
♀ ♂—10 (four of the females undergoing sex-reversal)
♀ ♀—13

The result of 29 one-sexed and 27 two-sexed pairs is quite close to expectation (28 ± 2.5). Among the total of 112 individuals we find 59♂ and 53♀ (the latter group including the sex-changing animals). The deviation from the mean is less than the single P.E. It can be considered, therefore, as well established, that *there was no sex transformation before the time of sex-differentiation.*

In discussing the fact of the dominance of the male sex in the free-martin, Lillie is inclined to consider the time-factor as an essential one. The observations of his students (Chapin, 1917 and Bascom, 1923), relating to the earlier development of interstitial cells in the testes and the later differentiation of the ovaries, seem to favor the theory of dominance of the male twin by earlier hormone production. Lillie admits, however, that other causes may also exist. In the case of our frog twins it is evident that the time-factor has not the suggested significance. The male-differentiating factor reaches the female gonads after the ovaries have acquired a relatively advanced stage of development. This means, that the female-differentiating factor is already in full action. In the resulting competition of both, the male-differentiator acts first as an inhibitor and later on as a suppressor of the female one. This is in strict agreement with the conclusions derived from the author's observations on hermaphroditism (1914; 1921). I think, that there can no longer be any doubt about *the antagonistic character* of these two factors.

Lillie (1923) and Bissonnette (1924) report on some earlier stages of the free-martins with rather highly developed ovarial structures. It seems not improbable that the female co-twins started with a typical female sex-differentiation. Such cases would lead to the same conclusions as the parabiotic frog twins. The assumption, that only the male is producing hormones in an early time, would apparently not be sufficient to explain the suppression of the active ovarial development. There seems to be no escape from the postulated sexual antagonism.

Some years ago Goldschmidt (1920) put forward the idea, that the sex-determining genes act like enzymes, stimulating the production of sex differentiating hormones. The term "hormones" taken in its proper sense, would indicate, that the sex-differentiators circulate in the blood and are spread throughout the entire body. The writer's previous studies on sex-differentiation in the frog seem to point in a somewhat different direction. In hermaphrodites the eggs are formed exclusively in the germinal epithelium, while the spermatogonia develop in the sex-cords. At the beginning of the sex-reversal from female to male those germ-cells situated closest to the sex-cords are the first to show characteristic changes and to migrate into the sex-cords. If we consider the behavior of the germ-cells as an indication of, or a reaction to, the nature of their environment, then we must attribute male differentiating properties to the sex-cords. On the other hand, from the fact that the germ-cells which were not attracted by the sex-cords, but which remained in the germinal epithelium, become eggs, it becomes evident that this epithelium includes female-differentiating properties. From such facts, which furthermore are supported by experimental data, I proposed the *theory of localized sex-differentiators* ("lokalisierte Innenfaktoren," 1914). These probably are comparable to Spemann's "Organisatoren."

The fact of independent sex-differentiation in genetically two-sexed twins favors again that localization theory. Regarding the secondary sex-reversal of the female part, the coöperation of hormones is not improbable. We may assume, either that part of the active substance elaborated in the sex-cords is carried away by the blood-stream, or that the differentiated testes produce a hormone, which is different from the substance inducing the first sex-differentiation. Yet, it is to be kept in mind, that—at least in the frogs—there is no definite proof, that the testes of the male twin act by way of the blood-stream. This question has to be discussed in connection with a detailed study of the homoplastic and heteroplastic twins, which will be published at a later date.

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BIOLOGICAL BULLETIN

THE EFFECT OF LACK OF OXYGEN ON SEA URCHIN EGGS.

ETHEL BROWNE HARVEY.

It has long been known that fertilized sea urchin eggs may live for some time in the absence of oxygen without division, and divide on readmission of oxygen (Loeb, '95). Since the rôle of oxygen is of fundamental importance in the life of the cell, I thought it might be of interest to make a study of the changes that take place in individual eggs during lack of oxygen at different stages of development, with special reference to the mitotic figure. The opportunity to procure excellent material for this study was given at the Naples Laboratory, where the perfectly transparent eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* may be procured during the entire year. It is with great pleasure that I thank Dr. Dohrn for his kindness and courtesy while working in the Stazione Zoologica, and also the Association to aid Scientific Research by Women for the use of their room.

The observations were made on eggs in a hanging drop in a modified Engelmann's chamber to which pure hydrogen[1] was admitted. The eggs were entangled in threads of platinized asbestos to absorb the last traces of oxygen, and they were stained in methylene blue in order to tell when the oxygen was completely gone. Under the conditions of the experiment, it was found that it took about twenty minutes for the oxygen to be entirely used up as indicated by the colorless condition of the originally blue stained eggs. The eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* gave practically the same results, and were equally good for observation, the eggs of *Echinus* being a little larger.

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[1] The hydrogen was prepared in a Kipp generator from zinc and sulphuric acid washed with alkaline permanganate and passed over platinized asbestos heated to redness in a quartz tube.

When the eggs are subjected to lack of oxygen immediately after fertilization (Fig. 1), the sperm aster forms and the two pronuclei approach just as under normal conditions, there being still sufficient oxygen for normal development. After about twenty minutes, however, the process is arrested owing to lack of oxygen. In some cases this takes place when the two pronuclei are still a little distance apart (Fig. 2). If kept in absence of oxygen, the sperm aster gradually fades out and the cell appears just as it did on fertilization (Fig. 3). This usually occurs in 40 to 60 minutes after admission of hydrogen to the chamber (room temperature 16°–18°). In most cases the female pronucleus is still visible even after two or three hours in hydrogen, but in some cells it cannot be discerned. In most of my experiments at this stage, the cells became irregular in outline after about forty minutes in hydrogen, sometimes earlier, and much crinkled, showing decidedly light and dark areas of protoplasm, and they remained in this condition, gradually increasing in intensity for two or three hours (Figs. 4 and 5). I am inclined to think that this is due, however, to other conditions than lack of oxygen since in two experiments most of the eggs remained uncrinkled and quite normal in appearance, and sometimes eggs under apparently normal conditions show the same peculiarities. They are somewhat similar to eggs kept in hypertonic sea water, although it cannot be due here to withdrawal of water since the same eggs in the same chamber later became smooth again.

When oxygen is admitted, the sperm aster reappears, in the same position where it disappeared, in the course of half an hour in the clear uncrenulated eggs, and development goes on in quite a normal fashion though all the stages are of course delayed. In the crenulated eggs, the dense protoplasm obscures the nuclear phenomena, and usually the mitotic figure is in the early metaphase before asters or nuclei are discernible (Fig. 6). Even though much crinkled and irregular, these eggs straighten out, become regular in outline and normal in appearance. The only abnormality observed in the development of the eggs which were subjected to hydrogen immediately after fertilization is in the first division plane sometimes coming in so as to divide the egg into two slightly unequal cells. In most experiments, the eggs developed into normal swimming larvæ next morning.

Eggs subjected to hydrogen at any later stage of development up to the formation of the metaphase spindle behave similarly to those described, and I have tried every stage. The astral rays gradually disappear under hydrogen, leaving the egg quite homogeneous; the eggs may become crenulated and irregular especially in the early stages. But when oxygen is admitted, the astral rays reappear and the eggs divide and develop normally except for the occasional unequal division of the first two cells. When the eggs are perfectly clear, the mitotic figure always reappears when oxygen is admitted *in exactly the same phase and position* in which it disappeared when oxygen was taken away.

When the eggs are kept without oxygen while in the full metaphase, in some cases the fibers become indistinct in about half an hour, but usually they remain distinct for an hour or more; in some cases the astral fibers have persisted longer than two hours. After the fibers disappear, the eggs become almost homogeneous, with perhaps a few darker areas (Fig. 7). When the eggs, stained in methylene blue are becoming decolorized by lack of oxygen, the blue remains last in the granules among the astral rays. This region is also a little more blue in the normal eggs stained in methylene blue than elsewhere, and is especially so in subsequent cleavages. In the very granular eggs of *Sphærechinus granularis*, the region around the spindle and asters is very blue while the periphery may be quite colorless.

When oxygen is admitted to the eggs which have been in a hydrogen atmosphere during the metaphase, the asters may reappear in the course of an hour in the same place where they disappeared (Fig. 8). In many cases, however, the mitotic figure assumes a peculiar appearance, a decided white streak running between the two asters and the astropheres are elongated in one axis (Figs. 19, 24). The white streak gradually disappears (Figs. 20, 21, 25, 26).

The eggs which have been subjected to lack of oxygen during the metaphase pursue the most peculiar development on readmission of air. Instead of dividing into two, frequently several asters appear (Figs. 9, 21, 28) and the egg divides quite irregularly. Sometimes one fairly large cell divides off (Fig. 10), sometimes several large lobes start to constrict off (Fig. 22), sometimes many very small fragments pinch off (Figs. 27, 28, 32, 33). Sometimes the constrictions complete themselves and form cells, sometimes they again fuse with the main cell body. The following stages are likewise irregular, the cells seeming to attempt all sorts of methods of righting themselves by trying first one plane and then another, meanwhile obliterating a plane already in existence.

These eggs seemed so abnormal in appearance that I at first threw them aside thinking that they had been harmed by some experimental conditions and that they were about to die. But much to my surprise, I found that many living larvæ were present in some of the chambers one morning. I have since watched many individual eggs pass through these most amazing stages, assuming the most bizarre appearance, cells of all sizes pinching off and again fusing in the most irregular fashion. But these same eggs succeed in righting themselves, and finally give rise to a number of equal cells, and go on with a regular cleavage resulting in normal blastulæ. Stages in the development of four such eggs are given in Figs. 7–18, 19–23, 24–31, 32–35. Even a very short exposure to the absence of oxygen (5 minutes) is sufficient to call forth these peculiarities when air is readmitted. A long exposure at any other stage previous to the full metaphase does not give this result.

If the cell has elongated preparatory to division after being in the chamber for about twenty minutes, that is, during absence of oxygen, if kept longer in hydrogen, it will again round out and if a division plane has started as it sometimes does, it will again become resorbed (Figs. 36–38, 39–41). In many cases a division plane has started to come in after the egg has been in the chamber 30 minutes, more than time for all the oxygen to have gone, and after the blue color of the methylene blue has gone but before the mitotic figure has faded out, but this is resorbed if kept in hydrogen. On readmission of oxygen, these eggs proceed with the same sort of irregular cleavages as those in the full metaphase, but result finally in normal blastulæ.

When the division plane has completely formed before total absence of oxygen, it remains, and when oxygen is admitted the cleavage goes on regularly, the egg dividing into two equal cells, then four, etc. The division planes which come in during a scarcity but not total absence of oxygen (*i.e.*, about 15 min. of hydrogen) are often somewhat crinkled and irregular (Fig. 42).

If the egg is subjected to lack of oxygen just before the second cleavage, when air is admitted, the same peculiar irregular divisions are formed as when exposed to hydrogen during the first metaphase (*i.e.*, just before the first cleavage), followed by fusion and irregular divisions, but resulting in normal blastulæ. If subjected to lack of oxygen at any stage between the completion of the first cleavage and the full metaphase of the second, the cleavage goes on normally when air is admitted.

These experiments show that sea urchin eggs may be deprived of oxygen at any stage, from just before the union of the two pronuclei until the completion of the first cleavage, and will remain in whatever stage they may be in, without further development, if kept without oxygen. Astral rays, whether of the sperm aster or the first cleavage asters at any stage, gradually disappear until the egg becomes quite homogeneous. The first cleavage furrow may start to come in during lack of oxygen, but is later resorbed. When oxygen is again admitted, the astral rays reappear in exactly the same position where they disappeared, the mitotic figure reappears in the same phase, and development proceeds. The reappearing mitotic figure and subsequent cleavage is perfectly normal at any stage except in those eggs which were kept without oxygen at the metaphase or a little later, when the reappearing mitotic figure is peculiar and the cleavages are quite irregular. Even the very abnormal looking eggs resulting from the irregular cleavages, however, right themselves and give rise to perfectly normal larvæ.

In Loeb's ('95) experiments, the eggs were exposed to lack of oxygen at only one stage, *i.e.*, soon after fertilization, and his results are quite in accord with mine for this stage. Demoor ('95) thought that in the cells of *Tradescantia*, one could make a distinction between protoplasmic life and nuclear life in their relation to oxygen, the latter going on in spite of lack of oxygen resulting in a continuation of mitotic division, while the protoplasmic life was arrested, no streaming of protoplasm taking place and no cell divisions. Such a distinction can certainly not be made in the case of the sea urchin egg, for practically all manifestations of life are inhibited by lack of oxygen, both nuclear and protoplasmic.

The fading out of the astral fibers and disappearance of the mitotic figure which resulted from exposure to lack of oxygen, are of interest in comparison with the early experiments of the Hertwigs ('87 and '90) with chloral hydrate, quinine and cold, and those of Wilson ('01) on ether. Perhaps the disappearance is even more complete in the case of absence of oxygen, for all traces of fibers, astral rays and astrospheres may be lost.

As indicated above, the lack of oxygen had practically no effect on subsequent development during any of the earlier stages of mitotic division. The astral rays and spindles come back in the same place and condition when oxygen is readmitted as they were before oxygen was taken away. It is only when oxygen is taken away during the full metaphase or a little after that the reappearing mitotic figure is different and the subsequent cleavages are aberrant, although even here normal larvæ are produced. This is contradictory to the results of E. P. Lyon ('02), who found that sea urchin eggs were most susceptible to lack of oxygen and to cold 10–15 minutes after fertilization. Although in my experiments, eggs were not in total absence of oxygen till 15–20 minutes after fertilization, they were at a lower temperature (*i.e.*, 16°–18° as opposed to 20° of Lyon) so that most likely his susceptible period was covered. The period that was most susceptible in my experiments was the metaphase and slightly later, a period which Lyon ('02, '04) found most resistant to lack of oxygen and cold but most susceptible to heat and most productive of CO_2. However, the criterion of susceptibility was different in his experiments and mine, his being the total number of survivors and mine being the microscopic appearance of individual eggs. The fate of the larvæ was quite precarious in my experiments, some lots of eggs going well and others under apparently identical conditions not surviving the following morning. When conditions are favorable, normal larvæ are produced after readmission of oxygen no matter at what stage in mitosis the eggs are subjected to lack of oxygen.

It might be of interest to compare briefly the results on oxygen consumption of the sea urchin eggs with my results on lack of oxygen. Warburg ('15) found that 10 minutes after fertilization, the oxygen consumption in *Strongylocentrotus* was six times that of the unfertilized egg and in the sixth hour was twelve times. Shearer ('22) by means of a special type of Barcroft manometer found that the oxygen consumption of *Echinus* eggs was eighty times as great during the first minute after fertilization as during the minute just before fertilization. The tremendous increase in oxygen consumption occurs at the time that the sperm comes in contact with the egg, before entrance. My experiments have not included this early stage owing to difficulties in technique. But it is interesting that Shearer obtained no special increase in oxygen consumption at the time of the union of the pronuclei, a stage which I found unaffected by lack of oxygen, whereas he did obtain an increase at the time of first cleavage (45–50 min. after fertilization), this corresponding roughly to the period just before first cleavage in my experiments when the effect of lack of oxygen was most pronounced (there being a very short time interval between anaphase and cleavage). Of course the period when most oxygen is consumed need not necessarily be the period when the lack of oxygen is most felt, but it is of interest that the two seem to harmonize to some extent.

The peculiar cleavage figures following readmission of oxygen to cells deprived of it during the metaphase are somewhat similar to those obtained by Wilson ('01) after recovery from ether, though much more pronounced. Just what is the meaning of the irregular cleavage, it is difficult to say, except perhaps that it is a readjustment after a very profound disturbance to the dividing cell due to lack of oxygen. There is no doubt that very extensive changes can be made in the early cleavages without affecting later development, as has been previously shown in many ways.

SUMMARY.

1. Observations were made on the living eggs of *Echinus microtuberculatus* and *Strongylocentrotus lividus* in a hanging drop

in a modified Engelmann's chamber. When the eggs are deprived of oxygen at any stage from fertilization to the completion of the first cleavage, development is arrested, but on readmission of air, continues and normal larvæ result.

2. When deprived of oxygen immediately after fertilization, the sperm aster gradually fades out and when air is readmitted, the aster reappears in the same position.

3. When deprived of oxygen at any later stage up to the metaphase, the astral fibers disappear but reappear on readmission of oxygen in the same position. Eggs have been as long as three hours without oxygen.

4. When deprived of oxygen at the full metaphase, the astral fibers disappear but sometimes not till after two hours. When air is admitted, the mitotic figure often appears as a white streak between two elongated astrospheres. Subsequent development is very irregular, often many asters appearing and cleavage planes dividing off large or small cells and subsequently often becoming obliterated, and other cleavage planes forming. After a while the eggs right themselves, giving rise to regular normal blastulæ and larvæ.

5. The egg may elongate and cleavage planes may start to come in at first during lack of oxygen, but the egg rounds out and the division plane is obliterated if kept longer without oxygen. These eggs proceed with irregular cleavages when air is admitted, but result in normal blastulæ.

6. If kept without oxygen at any stage after completion of the first cleavage to the metaphase of the second, normal cleavages result on admission of air.

7. If kept without oxygen just before second cleavage, when air is admitted, irregular divisions and fusions take place, but normal blastulæ result.

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PLATE I.

The drawings were all made with a camera lucida of living eggs under a Zeiss DD lens, ocular 2.

FIG. 1. Egg of *Echinus* immediately after fertilization.

FIG. 2. Same subjected to H for 20 min.

FIG. 3. Same after two hours in H.

FIG. 4. Usual appearance of egg after 40–60 min. in H, showing irregular outline and crinkling.

FIG. 5. Same after 1–3 hours in H.

FIG. 6. Same 50 min. after readmission of air.

FIG. 7. Egg of *Echinus* subjected to H at metaphase for 3½ hrs.

FIG. 8. Same egg 45 min. after air is readmitted.

FIG. 9. Same egg 25 min. later.

FIG. 10. Same 5 min. later.

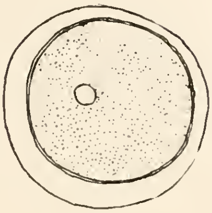
FIG. 11. Same 5 min. later.

FIG. 12. Same 30 min. later.

FIG. 13. Same 10 min. later.

FIG. 14. Same 5 min. later.

FIG. 15. Same 15 min. later showing fusion of cells.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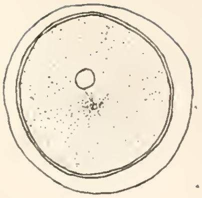
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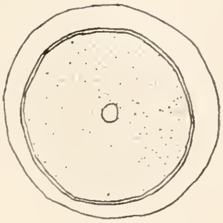
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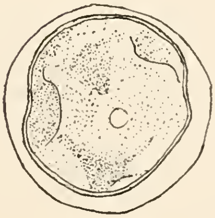
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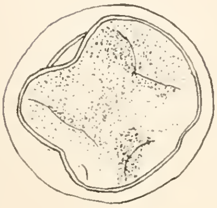
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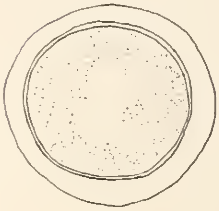
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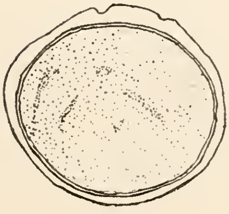
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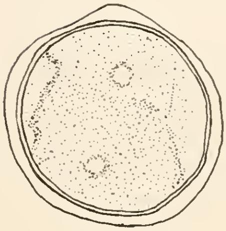
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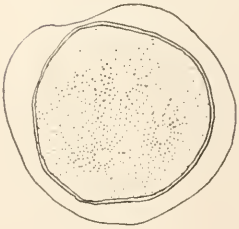
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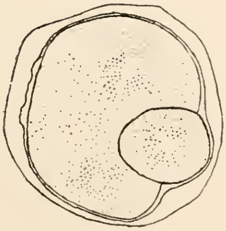
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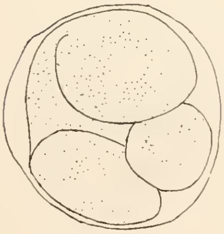
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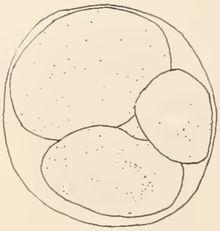
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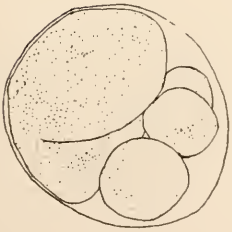
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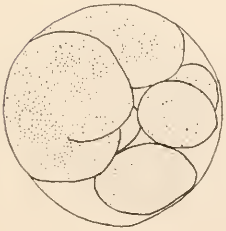
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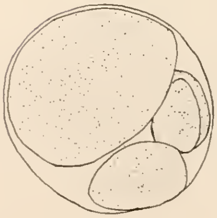
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ETHEL BROWNE HARVEY.

PLATE II.

FIG. 16. Same 20 min. later.

FIG. 17. Same 10 min. later.

FIG. 18. Same 15 min. later.

FIG. 19. Egg of *Echinus* subjected to H at metaphase for 1 hr. then air for 15 min.

FIG. 20. Same 20 min. later.

FIG. 21. Same 20 min. later.

FIG. 22. Same 20 min. later.

FIG. 23. Same 20 min. later.

FIG. 24. Egg of *Strongylocentrotus* subjected to H at metaphase for 15 min., then air admitted for 30 min.

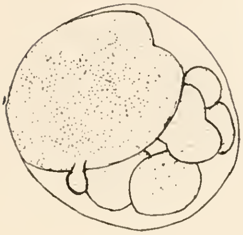
FIG. 25. Same egg 20 min. later.

FIG. 26. Same 20 min. later.

FIG. 27. Same 2½ hrs. later.

FIG. 28. Same 15 min. later.

FIG. 29. Same 15 min. later.



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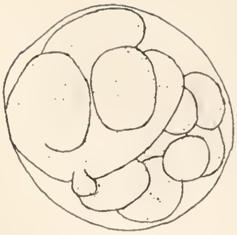

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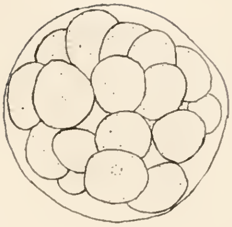

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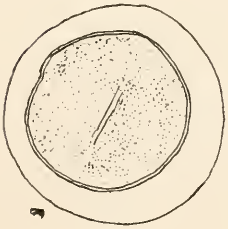

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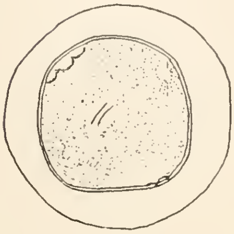

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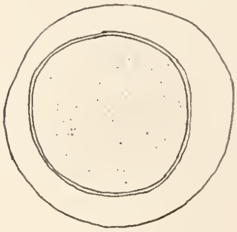

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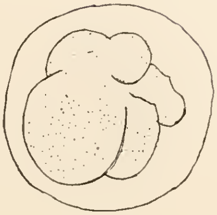

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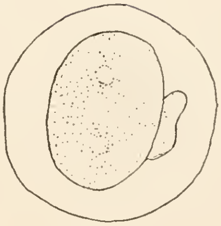

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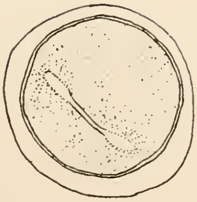

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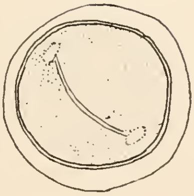

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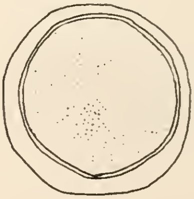

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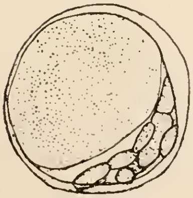

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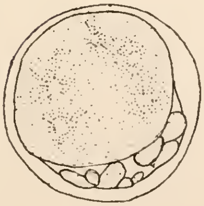

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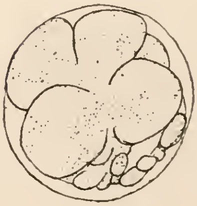

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ETHEL BROWNE HARVEY.

Plate III.

Fig. 30. Same 20 min. later.

Fig. 31. Same 1 hr. later.

Fig. 32. Another egg of *Strongylocentrotus* subjected to H at metaphase for 15 min., then air admitted for $3\frac{1}{2}$ hrs.

Fig. 33. Same 10 min. later.

Fig. 34. Same 30 min. later.

Fig. 35. Same 20 min. later.

Fig. 36. *Echinus* egg subjected to H for 20 min. in anaphase.

Fig. 37. Same egg still under H 15 min. later.

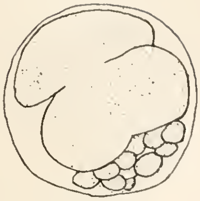
Fig. 38. Same egg still under H 30 min. later.

Fig. 39. *Echinus* egg subjected to H for 17 min. in anaphase.

Fig. 40. Same egg still under H a few min. later.

Fig. 41. Same egg still under H 30 min. later.

Fig. 42. *Echinus* egg divided while subjected to H for 15 min.



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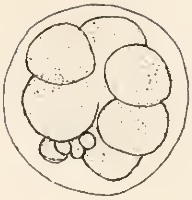

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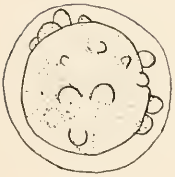

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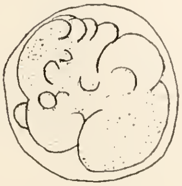

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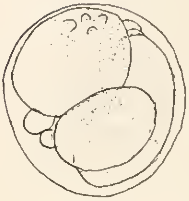

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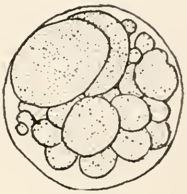

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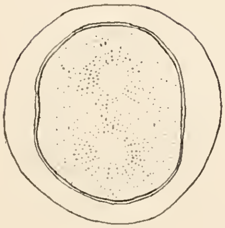

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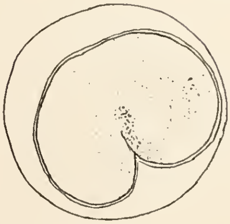

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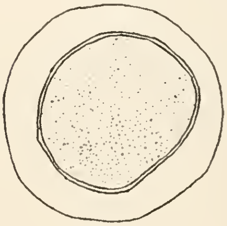

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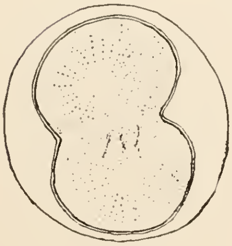

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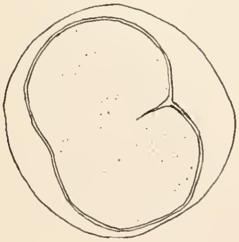

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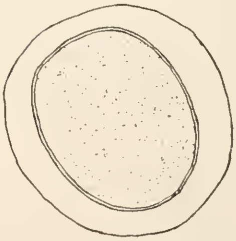

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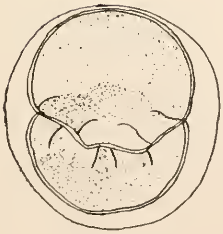

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ETHEL BROWNE HARVEY.

THE COMPOSITION OF WOODS HOLE SEA WATER.

IRVINE H. PAGE.[1]

There are no more fundamental quantitative data than those regarding the composition of the medium nurturing the organisms on which much of the study of biological laboratories is based. Analyses of the tank water in the Marine Biological Laboratory were therefore made using the methods briefly outlined below.

Calcium and Magnesium.—50 cc. samples of sea water were boiled with a little HCl to expel CO_2, cooled and the calcium precipitated as oxalate, the oxalate subsequently ignited and weighed as CaO. Magnesium was precipitated in the filtrate with Na_2HPO_4 in the presence of an excess NH_4OH, ignited and weighed as the pyrophosphate.

Potassium was determined in 1 cc. samples of water by the method of Kramer and Tisdall (1). This method essentially consists in the precipitation of potassium with cobalti-nitrite reagent, and titration of the precipitate after special washing with $KMnO_4$. The method has been found to be exceedingly accurate and therefore has many advantages over the old, classical and tedious, precipitation as the chloroplatinate or the perchlorate.

Sodium was estimated by a modified Kramer and Gittleman technique (2). We have found that modification of the original technique—which was designed for blood serum only and for this most useful—is necessary because both calcium and magnesium are precipitated practically quantitatively by the sodium reagent, and are titrated along with the sodium.

The modified method consists in preliminary precipitation of the calcium and magnesium with alkaline phosphate, centrifuging off the precipitate, concentrating the supernatant liquor to about 3 cc. on the water bath, cooling and adding 10 cc. of the alkaline potassium pyroantimonate reagent, then 3 cc. of redistilled (over KOH) ethyl alcohol drop by drop with vigorous shaking. This precipitation is carried out in 50 cc. conical bottom Pyrex centrifuge tubes; it is important that these be used. After one half hour standing the tubes are centrifuged and the procedure carried out as Kramer and Gittleman recommend.

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[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

Removal of the calcium as carbonate is unsatisfactory because of the very marked tendency of $CaCO_3$ to form supersaturated solutions. Gentle heating of the solution will, however, bring the carbonate down.

Oxalate precipitation seems quite satisfactory followed by phosphate for the magnesium precipitation, but here two steps are required and there is slight danger of carrying some oxalate along until the thiosulphate titration.

Using the above slight modification we have been able to get most accurate check analyses of known solutions of NaCl and $CaCl_2$. Kramer and Gittleman's method is a most useful and unique technique and seems to have a wide applicability for the direct determination of sodium.

As in the case of potassium we feel that the direct determination of sodium as compared with the determination by difference, as the combined sulphates, etc., has certain advantages.

Chlorine was estimated by the $AgNO_3$-$KCrO_4$ titration of Fr. Mohr.

Sulphate was precipitated as $BaSO_4$, ignited and weighed.

Phosphate was determined in 5 cc. samples of water by preliminary oxidation of organic matter with H_2SO_4 and H_2O_2 as described in more detail by Page (3), followed by the Benedict-Theis (4) technique. This technique consists essentially in the colorimetric estimation of a stable blue color developed by molybdic acid, hydroquinine and sodium bisulphite in the presence of a large excess of H_2SO_4. The following table gives the average of 4 analyses taken during the month of August 1926 from the tank water of the laboratory.

TABLE I.

ANALYSIS OF TANK WATER OF MARINE BIOLOGICAL LABORATORY.

Grams per 1000 cc. sea water at 20 degrees C.
Specific Gravity = 1.0180 (20 degrees C.).

		Millimoles.
Sodium	8.80	0.3826
Potassium	0.412	0.0105
Calcium	0.428	0.0107
Magnesium	1.3004	0.0534
Chlorine	18.350	0.5912
Sulphate	2.615	0.0272
Phosphate	0.002	—

DISCUSSION.

These analyses are in rather close agreement with those of Wheeler (5) made at Beaufort, North Carolina. The sodium and specific gravity are slightly lower than most of Wheeler's and Ditmar's (6) analyses. Whether the lower sodium is due to an error in one or other of the methods we cannot say.

The phosphate showed the marked seasonal variations first carefully investigated by Atkins (7). At times we could find no determinable quantity present. Atkins has shown that these variations are largely dependent on the plankton growth especially the diatoms.

F. W. Clark (8) gives a summary of most determinations that have been made of the composition of the oceanic salts.

SUMMARY.

1. The relative quantities of the more important salts in the Woods Hole, Massachusetts, sea water have been determined.

2. The Kramer-Gittleman technique for the determination of sodium in serum has been modified for the analysis of sea water. It has been shown that Mg and Ca interfere seriously when present in quantity and must be removed before the Na determination.

It is a pleasure to acknowledge my indebtedness to Dr. W. A. Perlzweig for his many valuable suggestions.

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THE OILS OF THE SEA URCHIN AND STAR FISH EGG.

IRVINE H. PAGE.[1]

The fatty fraction of the Echinoderm ova is particularly important because there is evidence that this fraction furnishes much of the energy requirement for the development of the egg. The sterol content of the eggs has been determined and a new sterol found in the star fish egg (*Asterias forbesii*) by Page (1). The sea urchin (*Arbacia punctulata*) egg was shown to contain a rather large quantity of true cholesterol. It seemed desirable now to examine the oil contained in these eggs, the results of which analysis are presented in this paper.

Oil Content.

The eggs were removed from the ovaries, strained, and strongly centrifuged to remove as much sea water as was possible. They were then placed in a flask and extracted first with an alcohol-ether mixture followed by repeated ether extractions for eight days at 40 degrees C. in a thermostat. Some of the excess ether was allowed to evaporate at room temperature and then acetone added in large excess in order to precipitate the acetone insoluble phosphatide fractions. After standing over night the mixture was filtered and the solvent from the filtrate allowed to evaporate at room temperature. The residue from this evaporation was placed in a $CaCl_2$ desiccator and the desiccator kept in the thermostat at 40 degrees C. for 4 days. The residual oily material was then subjected to the identification procedure detailed below. The phosphatide fraction was dried at 100 degrees C. and weighed.

Protocols.

Arbacia Oil.—The oil had a brownish red color and smelled strongly of lower fatty acids. The material had a heavy consistency and tended to lump together. It was freely miscible with CCl_4 chloroform, ether, and acetone. From its appearance on standing it seemed to behave somewhat as a semi-drying oil. 8.3 grams of oil was obtained from 183 million eggs. The iodine number as determined by the Wijs method was 146–148, and the saponification value (Kottstörfer value) approximately 606.0.

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[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

The I_2 value is suggestive of the marine animal oils such as Menhaden and Sardine oils, also the liver oils. The high saponification value confirms the results of the volatile acid determination presented in Table I. It also suggests that much of the oil represents fatty acids of low molecular weight.

The fatty acids volatile with steam were determined by the method of Dyer (2). Twelve million *Arbacia* eggs were used. The results of the steam distillation of star fish eggs is also included. 9,160,000 of these eggs were distilled. Three hours and forty minutes were required in both cases for the distillation.

TABLE I.

DYER METHOD FOR STEAM VOLATILE FATTY ACIDS OF *Arbacia* AND *Asterias.*

Fraction.	Amount of Distillate *Arbacia.*	Amount of Distillate *Asterias.*	Cc. $N/10$ NaOH Required to Neutralize *Arbacia.*	Cc. $N/10$ NaOH Required to Neutralize *Asterias.*
1	13.3	7	.346	.175
2	24.0	14.0	.72	.350
3	22.0	23.9	.44	.597
4	20.5	19.2	.246	.480
5	32.0	20.7	.384	.414
6	18.0	34.0	.18	.510
7	20.0	33.5	.20	.335
8	26.0	39.2	.26	.196
9	17.0	36.6	.17	.219
10	56.0	43.0	.56	.215
11	47.0	55.0	.25	.220
12	40.0	13.5	.24	.067
Total	335.8	339.6	4.001	3.778

6,000,000 *Asterias* eggs = 2.474 cc. $N/10$ NaOH.

6,000,000 *Arbacia* eggs = 2.000 cc. $N/10$ NaOH.

Since the *Arbacia* eggs = 75 μ in diameter and *Asterias* eggs = 104 μ in diameter.

Calculating *Arbacia* volume to that of *Asterias* = 2.527 cc. *N*/10 NaOH for 6,000,000 *Arbacia* eggs.

Asterias Oil.—An orange colored oil, non-drying, pungent odor very much like vanillin, which has about the consistency of olive oil. The I_2 number (Wijs) was approximately 110–115 and saponification number 318.8. The saponification number and the physical properties of the oil suggest that a much greater proportion of high molecular weight fatty acids are present in this oil than in that of *Arbacia*.

The *Asterias* oil is present in great abundance while *Arbacia* contains much less oil. This fact seems important. Distillation of *Asterias* eggs with KOH gives very appreciable amounts of H_2S. Very little resulted from the distillation of *Arbacia*.

PHOSPHATIDE FRACTIONS.

1.539 grams of acetone insoluble material was obtained from 183 million *Arbacia* eggs. Much of this precipitate was insoluble in boiling alcohol suggesting a high percentage of kephalin. The presence of sphingomyelin was indicated by the presence of a small amount of white precipitate on the addition of ether to the alcohol solution.

Acetone precipitates a large quantity of brownish white gummy material from *Asterias* egg extract. It was largely soluble in hot alcohol suggestive of a large proportion of lecithin. Further examination of this fraction has been deferred.

It is interesting to note that qualitatively large amounts of soaps were found especially in the *Asterias* egg.

SUMMARY.

1. The oil of the sea urchin egg (*Arbacia punctulata*) has an I_2 number of 146–148 and saponification value of approximately 606. The star fish (*Asterias forbesii*) egg oil I_2 number is about 110–115 and saponification value 318.8.

2. Steam distillation of the eggs by the Dyer method suggests along with the Köttstorfer number that the fatty acids of the *Arbacia* egg are of a lower order than those of the *Asterias* egg.

3. Qualitatively there appears to be more kephalin in the *Arbacia* egg and more lecithin in the *Asterias*. The *Asterias* egg

appears to contain more KOH decomposable sulphur compounds than the *Arbacia* egg.

4. Qualitatively large quantities of soaps were found.

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THE ELECTROLYTE CONTENT OF THE SEA URCHIN AND STAR FISH EGG.

IRVINE H. PAGE.[1]

The sea urchin (*Arbacia punctulata*) and star fish (*Asterias forbesii*) eggs have been the subject of very extensive morphological and physiological research. Since both of these types of investigation are intimately related to the chemical composition of the egg, the following analyses of the electrolyte content of these two Echinoderm ova are presented.

EXPERIMENTAL METHODS.

Arbacia eggs were removed from the ovaries, strained through cheese cloth, centrifuged until as much sea water as possible was removed. The egg mass was then almost solid. The surface layer was then quickly washed over with a few cubic centimeters of distilled water, not sufficient to cause cytolysis but enough to remove any slight amount of sea water remaining on the surface. A sample of the egg mass was removed to determine the number of eggs, the volume of the egg mass determined, and the mass dried at 100 degrees C. in a Freas oven until constant in weight. Samples of this dried material were then ashed in a platinum crucible in an electric crucible furnace, a few drops of HNO_3 being added to aid the ignition. The ash was weighed and dissolved in 5 cc. of $N/10$ HCl, made up to 25 cc., and the SiO_2 centrifuged out. Aliquot portions were then used for the special analytical procedures.

Calcium.—5 cc. of the filtrate were brought to about pH 6.0, 2 cc. saturated ammonium oxalate added and the centrifuge tube in which the solution was contained vigorously "rubbed down" with a stirring rod. After 12 hours the tube was centrifuged and the precipitate washed three times with hot water. The calcium was then determined by the usual ignition method as CaO and as an alternative procedure dissolved in 4N H_2SO_4 and titrated hot with $N/100$ $KMnO_4$.

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[1] Contribution from the Research Division of Eli Lilly and Company, Marine Biological Laboratory, Woods Hole, Mass.

Magnesium.—The filtrate from the above proceedure was then made strongly alkaline with concentrated NH_4OH, and an excess of a solution of K_2HPO_4 added, the solution allowed to stand overnight, the precipitate filtered off on ashless filter paper, washed with dilute NH_4OH, ignited strongly, and weighed as $Mg_2P_2O_7$.

Sodium.—The filtrate from the above procedures was then concentrated on a water bath to about 3 cc., made alkaline to phenolphthalein with sodium free 10 per cent. KOH, 10 cc. of potassium pyroantimonate reagent added and the procedure further carried out as recommended by Kramer and Gittlemann (1). Sodium cannot be determined directly in the presence of fairly large amounts of Ca or Mg because these two elements are precipitated by the pyroantimonate reagent and may be titrated as sodium.

Potassium.—2 cc. samples from the original filtrate were analysed for potassium by the method of Kramer and Tisdall (2).

Iron was estimated colorimetrically by the method of W. S. Allen, using salicylic acid to develop the color as described by Scott (3).

Sulphate determined as $BaSO_4$ following hot $BaCl_2$ precipitation.

Chlorides were titrated using the Fr. Mohr method.

Total Phosphorus and Lipoid Phosphorus was determined by the Benedict-Theis method (4) modified for egg analysis as described by Page (5).

Nitrates were estimated by the phenolsulphonic acid method ordinarily used in water analysis.

The above analyses were then carried out on filtrates prepared as follows: Samples of one million eggs were centrifuged to remove as much sea water as possible, made up to a 3 cc. mark on the centrifuge tube with distilled water, 3 cc. more distilled water added and the tube vigorously shaken to cytolyse the eggs. After standing 15 minutes 4 cc. of 20 per cent. trichloroacetic acid was added and approximately 0.2 grams of prepared bone black,[1] again shaken and allowed to stand 15 minutes. The mixture was then filtered through ashless filter paper and the filtrate used for analysis. The bone black should have removed the majority of the echinochrome.

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[1] Commercial bone black boiled with HCl, thrown onto a Büchner filter and washed until the filtrate was chloride free.

RESULTS.

Table I presents the average figure for the substance sought. In every case at least three analyses were done. The results are calculated on the basis of milligrams per one million eggs.

TABLE I.

ELECTROLYTE CONTENT OF ONE MILLION *Arbacia* EGGS.

Radicale.	Mg. per Million Eggs.	Millimols.	Milliequivalents.
Calcium	1.90	0.047	0.094
Magnesium	4.48	0.182	0.364
Sodium	1.301	0.056	0.056
Potassium	2.445	0.063	0.063
Iron	0.030	0.0005	0.0015
		0.348 Total cation	0.5785 Total cation
Sulphate	0.00046	0.00004	0.00008
Chloride	0.1864	0.0053	0.0053
Total phosphate	0.9064	0.0291	0.0873
Nitrate	Trace		
	1.109	0.0344 Total anion	0.0927 Total anion

11.26

The weight per million eggs of the material dried to constant weight at 100 degrees C. was approximately 0.124 grams, and the ash was 8.5–10 per cent. of the dry weight of eggs. The *Arbacia* egg averages 74.1 μ in diameter (Glaser (6)).

SiO_2 was not quantitatively determined but qualitatively rather large amounts were found present.

The distribution of the phosphorus between lipoid and inorganic is discussed in another paper (Page (7)).

The analysis of *Asterias* eggs was confined to the analysis of the trichloroacetic acid filtrates described above. Due to the greater size of the *Asterias* egg (103.6 micro for unfertilized eggs (Glaser (6))) only 250,000 egg samples were analyzed. The volume relation (4.1893) of the *Arbacia* and *Asterias* egg is 2.1–5.2 or one million—404,000 eggs, hence although for convenience

250,000 eggs were analyzed, the results are calculated to a basis of 404,000 eggs for comparative purposes. At least two analyses were done for each element determined but the results are statistically not so accurate as the *Arbacia* analyses due to the relatively small number of estimations. The relative values are probably more accurate than the absolute.

TABLE II.

ELECTROLYTE CONTENT OF THE *Asterias* EGG.

Radical.	Mgs. per 404.000 Eggs.
Calcium	1.487
Magnesium	2.101
Sodium	4.84–7.27
Potassium	6.77
Lipoid phosphorus	0.541
Acid soluble phosphorus	0.591

Due to the incompleteness of the data in Table II the milliequivalents are not calculated.

DISCUSSION.

In both the star fish and sea urchin egg the potassium is higher than the sodium although in the sea water of Woods Hole the sodium is much higher than the potassium. (See page (8) for analysis of Woods Hole sea water.) The Ca : Mg in the sea urchin egg is approximately 1 : 2 and in the star fish egg the Mg in the ratio is slightly lower than 2. Total phosphorus is slightly higher in the star fish than sea urchin but the distribution is about the same between lipoid and acid soluble. (See also page (7).) Sulphate in the sea urchin is very low.

Using the salicylic acid method for iron determination the values found for *Arbacia punctulata* are higher than those found by Warburg (9) for *Strongylocentrotus lividus.* We find 0.24 mgs. per gram of dried eggs whereas his value is only 0.01 mg. per gram.

The fact that the cation equivalent is much higher than the anion opens an interesting field for speculation. Presumably much of this excess cation is combined in the lipoid phase and some held by proteins. Certainly much of it must be osmotically inactive.

My thanks are due Dr. G. H. A. Clowes and Dr. W. H. Perlzweig for suggestions during the course of this investigation.

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THE REACTION OF HYDRA TO INANITION.

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During the past fifteen years the senior author has observed that Hydra, kept in laboratory aquaria to which food has not been added, decreased greatly in size. In this general or casual observation, it was further noticed that green Hydras survived under these adverse conditions for much longer periods than did Hydras of other species. This has been a general experience in this laboratory, though it stands in sharp contrast with Wagner's ('04) statement that "Green Hydras stand starving very poorly, usually perishing in two or three days" (p. 612). In later years it was observed further that the poorly fed Hydras would lose parts of their tentacles. Sometimes the full complement of tentacles has disappeared. During the last three years, we have been seeking to learn the conditions under which Hydras lose their tentacles.

The literature presents the following causes by which Hydras lose their tentacles. N. Annondale ('07), in studying *Hydra orientalis*, found a seasonal variation from four to six tentacles. During the hot season this Hydra has but four tentacles, while during the cold part of the year it has six tentacles. G. Entz ('12) observed that an infection with *Amœba hydroxena* may lead to a complete loss of tentacles. E. Reukauf ('12) observed that the ciliate *Prodon teres* caused Hydra to lose its tentacles. Again, the presence of another ciliate, *Kerona pediculus*, according to P. Schultze ('13), leads to hypertrophy of the tentacles. E. Schultz ('06) observed that hunger set up a process of dedifferentiation within the tentacles of Hydra; just as Huxley and De Beer ('23) found that hunger and toxins caused dedifferentiation and resorption of the tentacles of *Obelia* and *Campanulana*. Huxley and De Beer observed that this process of dedifferentiation and resorption might involve, not only the tentacles, but also part of the zoöid. Finally Berninger ('10) observed that, in response to inanition, Hydra lost its tentacles; but he did not see the manner in which they were lost.

We have found that Hydra is very susceptible to the impulses arising from scarcity of food. Hydras, at the time they are taken from the pond, present sometimes one or more fragmentary tentacles. That these do not represent mere accidental losses is suggested by the fact that in a flourishing population of Hydras one does not readily meet with such imperfect specimens, whereas when the population of a pond is on the decline specimens with incomplete tentacles are quite frequently encountered.

That Hydras react rather readily to hunger is further indicated by the observations of Wilson ('91). He records that "After the exhaustion of the food-animals—which takes place rapidly when the Hydras become very numerous—the Hydras are compelled to live wholly upon mud" or sediment at the bottom. In this sediment they find fragments of plant tissue and numerous minute Infusoria. The polyps, "after descending, usually gorge themselves with the sediment at the bottom." Welch and Loomis ('24) verified this observation. They found, however, "that the Hydras did not always thrive indefinitely on the sediment material dredged from the deepest parts of the lake," p. 227.

Realizing, then, the susceptibility of Hydra to hunger, we set to work to see if inanition was a factor leading to the loss of tentacles. This effort was made through the observation of living isolated individuals and the histological study of isolated individuals and controls.

Hydra has been observed taking into its body a tentacle's end as it clung to the food. This has been seen frequently. For example Mr. J. W. Nuttycombe saw a Hydra swallow the ends of two tentacles that had been involved in the capture of a Cyclops. After considerable struggle on the part of both the tentacles and the body-proper, these tentacles were thrown from the cœlenteron. So far as could be determined these tentacles had not been injured. In such examples, an effort to free the tentacle from the cœlenteric cavity is always apparent.

In contrast to this, Mr. M. C. Yoder and the senior author observed an isolated Hydra, that had been deprived of food, hold a tentacle within the cœlenteric cavity for twenty minutes. Throughout this period the tentacle remained quiet. There was in this case no effort to free the ingested tentacle. After twenty minutes, however, the tentacle was seen to be slipping out of the mouth. When freed the tentacle presented a constricted zone about which the peristome had held it during the period of ingestion.

The history of this tentacle has an interesting bearing upon our observations; for it shows that the tentacle was not one that had undergone necrosis prior to its having been ingested, since it showed no eroded tissue and eventually fully recovered, leaving no trace of the constricted region. This tentacle, therefore, had not suffered necrosis before being ingested. Marshall ('23) makes this observation: "Some brown Hydras were induced to swallow pieces of *Hydra viridis* by slipping the latter inside the carapace of *Daphnia*, but they were ejected along with the remains of food," p. 614. The suggestion may be taken that only dead Hydra-tissue might be freely ingested since Marshall, in this case, saw fresh Hydra tissue "ejected along with the remains of food."

But our observations do not lend support to this negative inference. Miss Edna McNally in 1923 observed an *Hydra viridis* "put the end of one of its tentacles into its mouth." The end of the tentacle could be clearly seen inside of the cœlenteron. After the tentacle had been kept in the cœlenteron about fifteen or twenty minutes, the Hydra dragged the shortened tentacle with a ragged end from the mouth, leaving part of it within the cœlenteron.

In addition to having actually seen an Hydra ingest a piece of its tentacle, we have observed a green specimen, which during sustained inanition had but recently lost parts of some of its tentacles, throw out from its mouth defragmented tissue of green Hydra. On one occasion, after such cellular material had been regurgitated, it was again ingested.

Thus, actual observation of living individuals has yielded evidence that Hydras, that have been deprived of food, feed upon their tentacles.

Further evidence was secured through isolating two series of individuals. By depriving one of these series of food and feeding the other series well, we have secured some interesting results.

On March 14, 1925, six individuals were isolated in six watch glasses and given no food. Another lot of six were isolated. These were used as controls and were kept supplied with food in the form of *Cyclops* and daphnids. The observations were kept up until April 13, during which time, one of the controls had died (March 22) but none of these controls had lost part of their tentacles. Except for the one specimen dying, the controls at the end of the period of observation were in excellent condition and showed no negative features. The starving specimens presented a marked contrast to the control series for by March 22 two specimens had died. Of these two that had died, one had lost more than two thirds the length of each of its tentacles the day before it had died. The four other specimens, by April 14, had lost parts of their tentacles. The most conspicuous individual in the starved series was designated specimen six in our records. Figure 5, *A*, represents the contour of this animal when expanded at the time of isolation. On March 24 it showed, in the expanded condition, that its tentacles were shorter and knobbed on the end (Fig. 5, *B*). After that not much change could be noted, except that the knobbed condition of the tentacles had disappeared, until March 30. Then it was observed that a great amount of the tentacles had been lost (Fig. 5, *C*). On March 31, but mere stumps of the tentacles remained (Fig. 5, *D*). The specimen on April 1, showed the bud of one tentacle making its appearance. By April 8, tentacles relatively as long as those shown at *A* in Fig. 5 were present. During the period of inanition the body, as a whole, had been reduced in size so that by April 10 the regenerated tentacles were relatively longer than those which were involved at the beginning of the observation (Fig. 5, *E*). On April 9, some parts of this second crop of tentacles had been eaten. On April 14, the specimen died.

In the above observations, no effort to control the factor, presented by the accumulation of toxic metabolic products, was made. It is quite possible that such effort need not have been made, for the fed Hydras had grown in size and, therefore, must have thrown a greater amount of toxic material into the water than did the unfed specimens. In addition to this, the presence of the *Cyclops* and daphnids added much to the concentration of metabolic toxins in the water of the fed specimens as compared to the water in which the unfed specimens were kept. However, a second series of observations was made. In this series, five specimens were isolated and carried through unfed while five isolated specimens were fed to serve as controls. Each day the water in all watch-glasses containing Hydras was areated by blowing air through it and every twenty-four hours or every forty-eight hours the old water was drawn off and fresh spring water added. In these instances, we got just as conspicuous contrast between the unfed series and the fed series as has been described for the series running from March 14 to April 13. The most pronounced example, obtained in this manner of Hydra's reaction to inanition, was obtained from specimen 4 of a series begun October 6, 1925, and ending October 30. A camera lucida drawing was made of this specimen as it hung from the surface of the water in the watch-glass, October 6 (Fig. 6). Three days later the camera lucida drawing shows that there had been a marked reduction in the size of the specimen (Fig. 6). On October 11, the specimen presented only mere stumps of tentacles (Fig. 6). Camera lucida outlines made daily showed that those tentacle stumps gradually grew until by October 22 the camera lucida outline reveals that the now smaller Hydra had regenerated seven tapering tentacles (Fig. 6). The next day, however, one of these tentacles had disappeared (Fig. 6). A second tentacle was missing October 24 (Fig. 6). Two days later the camera lucida drawing indicates that the specimen had lost the greater part of each of its tentacles (Fig. 6). In this condition the specimen remained as a listless individual for three days and then died.

The above specimens were examined daily for the presence of parasites such as *Amœba* and other protozoa and were thus determined to have been free of such infection. The abruptness, however, with which a part of a tentacle or a whole complement of tentacles would be lost did not suggest that these tentacles disappeared through attack by parasites or by degeneration and resorption. It suggests rather that the specimens had been feeding upon their tentacles.

The histological evidence supports the idea that dedifferentiation, degeneration and parasites were not involved.

Specimen "369—2—a" had been isolated in a hanging drop until it had lost its tentacles. The slide made from this specimen bears a complete series of sections in which the shortened tentacles show no erosion such as *Amœba* and other parasites cause. Moreover, all of our slides show no dedifferentiation processes. We have slides that show wound scars at the end of tentacular stumps but in which the ectoderm is laid down as a continuous sheet about the tip (Example "370—2").

But our histological evidence leads further to the conviction that tentacle-material is ingested and digested. Mr. Looper, of this laboratory, has two individuals sectioned, in one of which the sections show a series of sections of three fragments of tentacles lying within the cœlenteron, while the other shows but one fragment of tentacle lying within the cœlenteron (Looper's slides "2 ovum la," and "Hydra Ovum 11").

The transverse sections of the tentacular fragments, that are to be found in sections of polyps that have suffered inanition, show a diploblastic pattern but present very poor detail. In Fig. 4 we have a transverse section of an ingested tentacular fragment. The region of the endodermal epithelio-muscular cells is most greatly broken down (Fig. 4, *b*). What we take to be residia of secreting endodermal cells are indicated at *a*. The ectoderm seems to be least attacked by the digestive fluids of the cœlenteron. All elements here, too, have been greatly shrunken.

Not only do the histological specimens show the tentacular fragments lying within the cœlenteron but they indicate that the material, thus taken in, is digested and absorbed.

This was further checked by fixing specimens by the pond in which a vigorous population of Hydras was growing and contrasting the histology of these with that of the isolated specimens that had been kept under daily observation in the laboratory. Slides, bearing material taken from the vigorous population of a pond, show no Hydra-tissue within the cœlenteron and very few, if any, nematocysts within the epithelio-muscular cells of the endoderm. In contrast to this, the histology of specimen "375" is significant. This specimen was isolated in a Petri dish, containing spring water, at 3:30 P.M., October 28, 1921. At 9:25 A.M. the next day it was found to have had but the merest stumps of tentacles. Our slide shows the tentacular stumps involving but a few sections of the series, thus recording their small size. Hydra-tissue, being digested within the cœlenteron, is found in these sections. While within the endoderm's epithelio-muscular cells are *many* nematocysts.

Another specimen, "369—1," was kept isolated within a hanging drop for three days and examined daily. At the end of this period, it was found to be in a listless condition and to have very short tentacles. This Hydra, in the fixed and sectioned condition, revealed many nematocysts within the cœlenteron and food-vacuoles of the epithelio-muscular cells of the endoderm.

Specimen "380" was isolated in a test tube. This tube was kept partly submerged in the pool from which the Hydra had been taken. Daily inspection revealed that on the seventh day the tentacles were blunt, their ends ragged, no amœbas or other parasites present. The series of sections on our slide 380 shows almost wholly digested hydra-tissue within the cœlenteron, there being left, as yet, naked nuclei, at one side of the dissolving mass, and a cnidoblast, in bad condition, surrounding a "stinging" nematocyst. In addition to this there are many nematocysts in various phases of digestion within endodermal cells.

These observations indicate that, not only are the protoplasmic constituents of the ingested fragments of tentacles digested and absorbed, but that even the nematocysts are likewise appropriated as food.

The fact that these structures can be digested by Hydra is interesting. Glaser and Sparrow ('09) found that "in the case of peptic digestions, all the tissues except the nematocysts, were dissolved," p. 362. But it has since been observed that parasitic ciliates and amœba can digest the nematocysts of Hydra. The histology of Microstoma's enteron or "intestine" greatly resembles that of Hydra and it does not digest the nematocysts but hands them over for offensive or defensive purposes to the mesenchyme. Hydra's immediate demand, during inanition, is neither defensive nor offensive but nutritional, so it digests the nematocysts. Fig. 1 shows a nematocyst from which the cnidoblast has already disappeared. Except for the lacking cnidoblast the nematocyst, as it lies within the food vacuole of an endodermal epithelio-muscular cell, does not present a markedly unusual appearance. In Fig. 2, however, the enclosed thread of the nematocyst displays marked shortening and the nematocyst, as a whole, takes the stain less readily. In Fig. 3, we have a contrast presented between a nematocyst that has been almost digested and one lying in the usual position in the ectoderm. It is seen here that the nematocyst within the endodermal cell has been almost wholly dissolved. Eventually no trace of the ingested nematocyst is to be found. The hungry Hydra, therefore, consumes not only the readily digestible tissues of its tentacles but also the nematocysts. From the material, thus obtained, energy is derived upon which the life of the polyp is tided through a period of inanition and by which the polyp is enabled to develop a new complement of tentacles.

An interesting feature of this process is presented in the fact that the stumps of tentacles receive relatively much more of the ingested tentacular material than do other parts of the body Our slide "375" shows this clearly. This slide bears in series the sections of an Hydra that had been isolated in a Petri dish in the laboratory for eighteen hours. At the end of this period but small stumps of its tentacles had remained. In this series of transverse sections there are ninety-five sections.

The tentacles involve about twenty of these (10 microns) transverse sections. The endodermal cells in the sections that show the bases of the tentacle-stumps are heavily charged with food-vacuoles that contain nematocysts and hydra-cells. The endodermal cells of the tentacle-stumps have in this instance much more food supplied them than do the endodermal cells of the body proper. This is in contrast to what ordinarily takes place. If a complete Hydra be fed and later sectioned, the endodermal cells of the tentacles will be found to have relatively fewer food-vacuoles than the endodermal cells of the body-proper. So here again we have it suggested that in a diploblastic animal local needs must be locally met. In Hydra, having but two tissues there can be no circulatory medium. Therefore, when material is needed for the regeneration of tentacles it cannot be taken up by the general endoderm of the body-proper and then as lymph or plasma sent to the tentacular bases, but the material must be handed directly, in the form of food, to the endoderm of the tentacle-bases.

SUMMARY.

Hydra, as a diploblastic animal, can have no circulatory medium. Perhaps for a similar reason it can have no storage tissue such as fat. Green Hydras seem able to fall back upon the surplus foods of its zoöchlorellæ, for, contrary to some observers, it has been our experience that *Hydra viridis* is much less influenced by inanition than other species.

In all species, if the inanition be prolonged and the individuals be kept free from parasites and concentrated toxins, each specimen not getting food, will begin to feed upon its tentacles. First the ends of the tentacles are bitten off. If now food is yet with-held from the Hydra, it will feed upon its tentacles until but the merest stumps are left.

The ingested tentacles will be digested. Even the nematocysts (both types) will be taken into food-vacuoles within the epithelio-muscular cells and be completely digested and absorbed.

After the Hydra has thus fed upon and appropriated its tentacles—except for mere stumps that stand about the peristome—the bases of the amputated tentacles will regenerate and the Hydra, now reduced in size, will possess a new group of tentacles.

In feeding upon the tentacles that have been ingested, the endoderm of the stumps of tentacles appropriate relatively more of the ingested tentacle-material than is appropriated by the endoderm of the body proper. This appears to be correlated with the fact that regeneration is to take place in the tentacular stumps.

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EXPLANATION OF PLATE.

FIG. 1. Interlocking secreting (*a*) and epithelio-muscular (*b*) cells of the endoderm. *b* bears a food vacuole (*c*) within which is a nematocyst that shows little distortion. × 1500.

FIG. 2. An epithelio-muscular cell of endoderm bearing a food vacuole (*a*) within which is a nematocyst that shows advanced internal disintegration. × 1500.

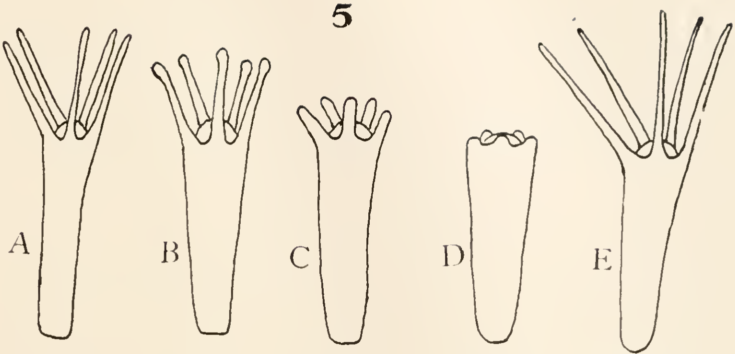
FIG. 3. An epithelio-muscular cell of endoderm with adjacent ectodermal cells. A greatly broken down nematocyst within a food vacuole (*a*) is contrasted with a nematocyst in the usual position within the ectoderm (*b*). × 1500.

FIG. 4. One of the transverse sections of a tentacular fragment that lay in the cœlenteron of a sectioned Hydra from which food had been withheld. (*a*) residia of secreting endodermal cells; (*b*) residia of epithelio-muscular endodermal cells. × 750. From H. L. Firebaugh's slide "No. 15. 34.7–85.8."

FIG. 5. Series of free-hand outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.

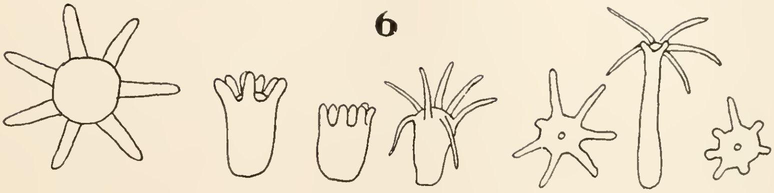
FIG. 6. Series of camera-lucida outlines to indicate changes that had taken place in an individual *Hydra* from which food had been withheld.

5



March 14 to March 23.
March 24 to March 29.
March 30.
March 31.
April 8.

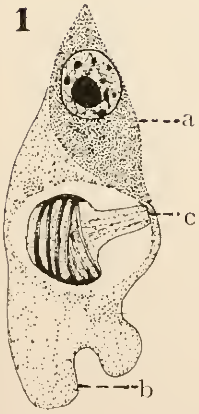
6



Oct. 6. Contracted Aboral Aspect.
Oct. 9. Contracted.
Oct. 11. Contracted.
Oct. 22. Expanding.
Oct. 23. Contracting.
Oct. 24. Expanded.
Oct. 26. Contracted.

Oct. 30. Dead.

1



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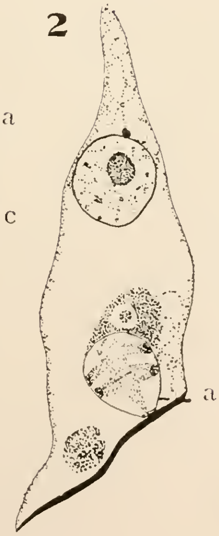

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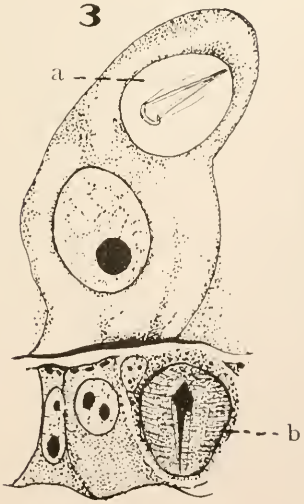

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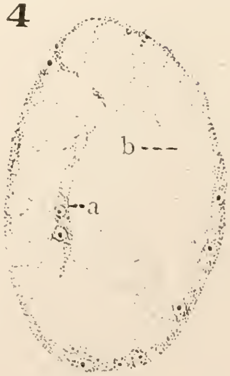

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CILIATED PITS OF *PRORHYNCHUS STAGNALIS.*

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The specimens used in this study were collected in the early months of the year in a very small, slowly running stream, the outlet of a spring, which is located about a mile west of the University of Virginia. They occurred in greatest abundance from twenty-five to forty feet below the spring, in the soft, oozy, decaying organic matter at the bottom of the stream. *Prorhynchus applanatus* was always found in equal, if not greater numbers, along with *Prorhynchus stagnalis*.

Some of the grass, weeds, and algae growing in the stream were also taken along with the sediment, and the whole collection placed in a large aquarium dish and left to stand for from twelve to twenty-four hours. The rhabdocœles appeared at the edge of the aquarium, near the water's surface, sometimes just beneath the surface film. When a film of bacteria developed at the surface of the water, the animals soon died, seldom being obtained five days after the collection had been in the laboratory. By keeping some of them in small dishes in spring water with some small amount of the decaying matter collected in the stream, and by adding fresh water every three or four days, specimens were kept alive for a maximum period of two weeks.

The body of *Prorhynchus stagnalis*, when moving through the water, is very slender or thread-like, being about the same width all along the body-length. When at rest, the posterior half is slightly broader, the average length of the body being about 6 mm.

The anterior region of *Prorhynchus stagnalis* is the exploratory region of the body. For, in response to a disturbance, the region of the body, anterior to the ciliated pits, of an animal that is quiet or of one that is creeping, is laterally expanded (Fig. I, *l.e.*) and swayed to and fro; while in addition to this the cilia of the pits, during disturbance, are set into active motion. The cilia of the ciliated pits are quiet when the animal is not excited or disturbed.

For histological study, specimens were fixed with greatest success in Bouin's fluid, cut from five microns to seven microns thick, and stained in iron hæmatoxylin, with Bordeau red or eosin as a counter stain. It was found best to handle the specimens in very small numbers at a time. While fixing, it was also found advantageous to keep the animal under the slight pressure of a cover glass to prevent the curling of the body. The best stain for differentiating nervous tissues was Mallory's connective-tissue stain, fixation being in Zenker's fluid. The nervous tissue is stained blue in this case.

Acknowledgment must be made to Dr. W. H. Taliaferro for a box of slides of *Prorhynchus stagnalis* which he left with Dr. Kepner, and which were of great aid in this work.

It is necessary to consider the central nervous system with which the two ciliated pits are associated before discussing the latter. There are two dorsal ganglia just anterior to the opening of the muscular pharynx as it lies at rest within the pharyngeal sheath (Figs. II and III, *d.g.*). These ganglia are connected across the pharyngeal sac by a relatively broad commissure (Figs. II and III, *d.c.*). Posteriorly from the dorsal ganglia extend latero-ventrally two nerves, which cannot be traced beyond the beginning of the enteron (Figs. II and III, *p.n.*). Anteriorly from the dorsal ganglia extend latero-ventrally two other nerves, which lie in contact with the mesial surfaces of the ciliated pits, and passing beyond the ciliated pits branch in the extreme anterior portion of the body (Figs. II and III, *a.n.*). There is another set of nerves connected with the pharynx, the exact path and relation of these nerves to the ganglia and pharynx having not been definitely determined.

From Kepner and Taliaferro's ('16) paper on "Organs of Special Sense of *Prorhynchus applanatus Kennel*," we find this comparison of the ciliated pits in *Microstoma caudatum* and *Prorhynchus applanatus:*

"As stated before, we consider the ciliated pits the chief organs of special sense. These organs open on the ventral side and are disposed laterally and ventrally to the thick nerve commissure which joins the two dorsal ganglia. . . . When the animal is crawling upon a surface, it seems to make numerous exploratory movements by raising and lowering the anterior sixth of its body. Thus we have a crawling animal with ventrally disposed ciliated pits which makes its exploratory movements by raising and lowering the anterior end of its body. It is well to compare the conduct of this animal with that of *Microstoma caudatum Leidy* with reference to the position of their respective ciliated pits. *Microstoma caudatum* is a free-swimming animal and has laterally disposed pits. As we have shown ('12) this animal makes exploratory movements by moving its anterior end from side to side. Likewise, we gave experimental evidence to show that these exploratory movements were made in order to test the surrounding medium. Thus we see by the comparison of the two rhabdocœles that the method in which they test the surrounding water conforms to the position of their ciliated pits."

So much for *Microstoma caudatum* and *Prorhynchus applanatus*. Kepner and Taliaferro in *Microstoma caudatum* dealt with a form that was primarily a free swimming one; in *Prorhynchus applanatus* with a form that was primarily a creeping one. *Prorhynchus stagnalis* usually creeps. It is, however, provided with mucous cells at the posterior end of its body by means of which it can attach itself to the substratum. From this stationary point it can move its body about in an almost complete circle, sometimes adhering closely to the surface, but frequently lifting the anterior third of its body, moving it either from side to side or backwards and forwards. Therefore, it would be natural to expect its special sense organs to be ventro-laterally disposed—and such happens to be the case. In *Prorhynchus stagnalis* is, therefore, presented a form that sometimes creeps and sometimes swims freely. So it is of interest to observe that when it is creeping the anterior end, through flattening, applies the ciliated pits to the substratum much as *Prorhynchus applanatus* does; whereas when the animal moves or sways through the water, its anterior end, through becoming rounded, places the ciliated pits more laterally as they are always placed in *Microstoma caudatum*. It has been thus observed that the position of the ciliated pits of *Prorhynchus stagnalis* also conforms with the diverse habits of *Prorhynchus stagnalis*.

The pits are directed obliquely posteriorly and mesially, there being a decided curvature in the "neck" of the pit.

In the histological structure of these pits we define three regions; the neck or transitory region (*t*), the sensory region (*s*), and the glandular region (*g*) (Figs. IV, V, and VII). All the components of the pits, with the single exception of the muscle fibers (Figs. IV, VI, and VII, *l.m.*, *m.m.*), which are attached to the pits, are considered to be of ectodermal origin. This assumption is based upon the fact that Kepner and Taliaferro ('12) in their study of the ciliated pits of *Microstoma caudatum*, clearly showed that in a newly-forming individual, both the glandular and sensory cells developed as modifications of an invagination of the general body epithelium or ectoderm (Figs. IV, V, and VII, *g.b.e.*). The pits of *Prorhynchus stagnalis* measure from twenty-five to thirty-five microns in length and from twelve to fifteen microns in width, (excluding the glandular cell, which is about two thirds of the length of the pit proper), being narrower near the mouth in the fixed condition on a slide than at the fundus of the pit. This latter condition, that of the narrowness of the mouth of the pit, is in sharp contrast to the contour of the functioning pit.

With the exception of the glandular region, the ciliated pit is, like the rest of the body epithelium, a syncytium, with its nuclei specialized to conform to their respective functions. The presence of a glandular cell makes even stronger the belief that the pit is a gustatory organ.

The nuclei of the ciliated, transitory epithelium are eight in number, a double row of four nuclei, one row being disposed obliquely dorso-ventrally, forming a part of the lateral wall of the pit, while the other row forms a similar mesial wall. These nuclei have an elongated, ovate contour with the axis of each nucleus in a plane that is at right angles to the axis of the pit, and bent to conform to the curved wall of the pit (Figs. IV, V, VI, and VII, *t*). They are densely granular, and the aspect of the possible fifth pair of nuclei nearest the mouth of the pit gradually fade into the appearance of nuclei of the general body epithelium.

There are also two rows of sensory nuclei, four in each row, and placed in the same plane as the nuclei of the transitory cells (Figs. IV, V, VI, and VII, *s*). On the inner walls of the pit next to the sensory nuclei are two rather densely granular ridges, (Figs. IV, and VI, *r.*), presumably for the receiving of stimuli. These ridges are devoid of cilia, though the remaining inner surface of the pit is highly ciliated. Due to the difficulty in differentiating nervous tissue very clearly through any of the simpler staining methods, it was impossible to distinguish any special nerve endings. The main anterior nerves from the dorsal ganglia were closely applied to the outer surface of the pit along its mesial side only (Figs. IV, V, VI, and VII, *a.n.*). Until better stained sections can be studied, the matter of nerve application to the pit will have to rest with this general statement.

At the base of the lumen of the pit, and connected with it only through small pores (*p.*) which pass through the syncytial wall of the pit on the anterior angle of the fundus lies a large uni-cellular gland (Figs. IV, V, VI, and VII, *g.*). Its contour is very irregular, the cell giving off a number of processes. There are two interesting features to this glandular cell not noted in that of *Prorhynchus applanatus.* One concerns the characteristic bent, ovate contour of the nucleus of this cell (Figs. IV, V, and VII, *g.n.*), with clearly defined chromatin granules. The other interesting fact concerns the presence of a simple drainage system in the form of intra-cytoplasmic canaliculi (Figs. IV, V, VI, and VII, *cn.*), which pour the mucus-like secretions of the cell through the pores in the syncytial wall into the pit (Figs. IV, VI, and VII, *p.*). These canaliculi have been observed both in an empty state and filled with materials of secretion.

Connected with the ciliated pits are several non-striated muscle fibers, arising both laterally and mesially, and passing closely along the surface of the glandular cell. There is only one fiber attached to the lateral wall (Figs. IV and VII, *l.m.*), but at least three fibers attached to the mesial wall of the pit (Figs. VI and VII, *m.m.*).

Summary.

1. The correlation between the position of the ciliated pits in *Prorhynchus stagnalis* and the way in which the anterior portion of the body makes its exploratory movements is met in this rhabdocœle in the same manner as in *Microstoma caudatum* and *Prorhynchus applanatus.*

2. The ciliated pits of *Prorhynchus stagnalis* are composed of a

definite number of cells, seventeen each. In spite of the fact that *Prorhynchus stagnalis* has no specialized sensory tissue in the form of a pair of simple eyes, *Prorhynchus stagnalis* is considered being more highly developed in that a much larger number of cells go into the formation of the pits than in both of the sensory organs of *Prorhynchus applanatus.*

3. The ciliated pits of *Prorhynchus stagnalis* are sharply differentiated into three regions: transitory, sensory, and glandular.

4. There are two interesting features to the glandular cell not noted in *Prorhynchus applanatus:* (1), the characteristic curvature of the nucleus; and (2), the intra-cytoplasmic canaliculi.

5. The ciliated pits of *Prorhynchus stagnalis* possess a definite musculature, attached to both their mesial and lateral walls.

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EXPLANATION OF FIGURES.

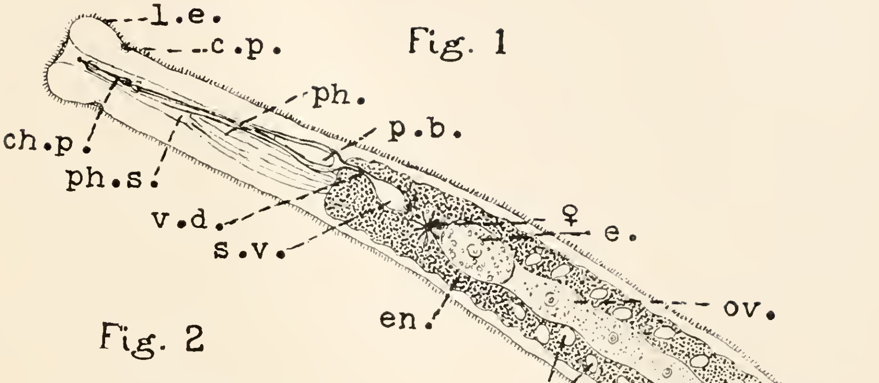
Plate I.

Fig. 1. Ventral aspect of Prorhynchus stagnalis to show especially the lateral expansion (*l.e.*) of the exploratory region anterior to the ciliated pits (*c.p.*). Chitinious penis (*ch.p.*); pharyngeal sac (*ph.s.*); pharynx (*ph.*); penis bulb (*p.b.*); enteron (*en.*); seminal vesicle (*s.v.*); vas deferens (*v.d.*); egg about to be liberated (*e.*); ovary (*ov.*); testes (*t.*); opening of female reproductive organs (♀); (Modified from Von Graff).

Fig. 2. Dorsal aspect of anterior third of body to show relation of ciliated pits (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); right dorsal ganglion (*d.g.*); right anterior nerve (*a.n.*); right posterior nerve (*p.n.*).

Fig. 3. Right lateral aspect of anterior third of body to show relation of ciliated pit (*c.p.*) to central nervous system. Pharyngeal sac (*ph.s.*); pharynx (*ph.*); dorsal commissure (*d.c.*); dorsal ganglion (*d.g.*); anterior nerve (*a.n.*); posterior nerve (*p.n.*).

Fig. 1


l.e.
c.p.
ch.p.
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ph.
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v.d.
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Fig. 2

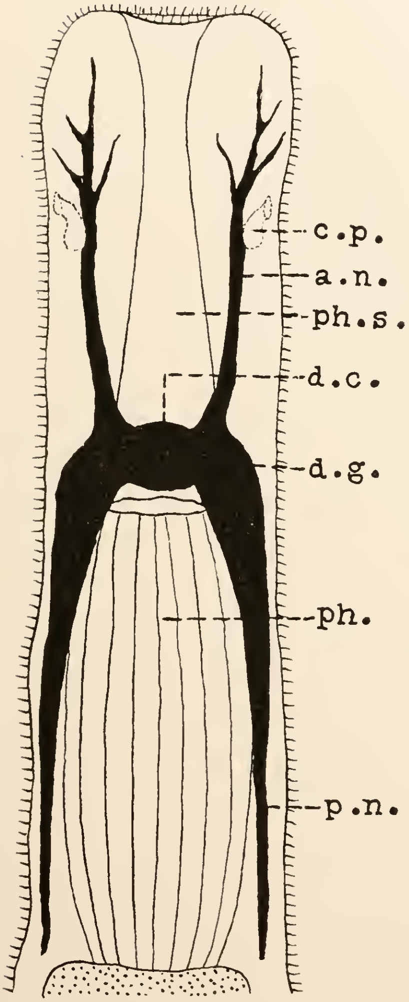
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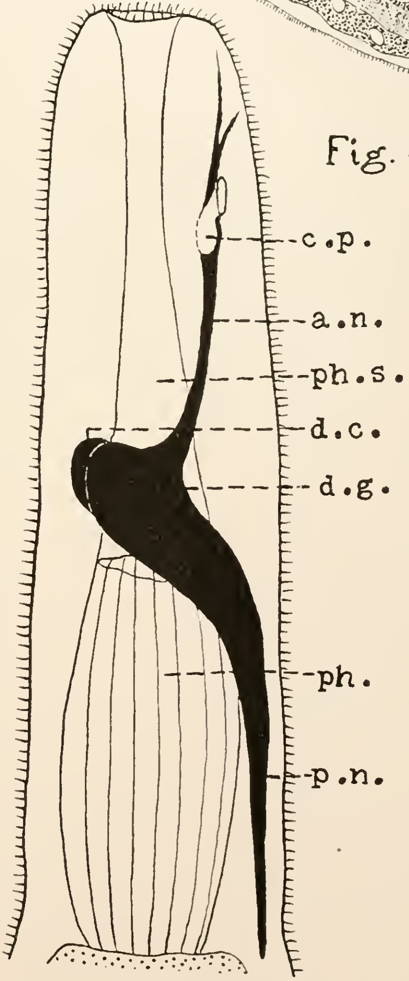
Fig. 3


c.p.
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d.g.
ph.
p.n.

PLATE II.

FIG. 4. Longitudinal section through a left ciliated pit. General body epithelium (*g.b.e.*); transitory nucleus (*t.*); non-ciliated ridge (*r.*); sensory nucleus (*s.*); pore of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); nucleus of gland cell (*g.n.*); lateral muscle fiber (*l.m.*); × 1500.

FIG. 5. Longitudinal section through a left ciliated pit, especially to show the row of four sensory nuclei (*s.*); General body epithelium (*g.b.e.*); anterior nerve (*a.n.*); secretion in pores of gland cell which lead into lumen of pit (*se.*); canaliculi (*cn.*); gland cell (*g.*); nucleus of gland cell (*g.n.*); × 1500.

Fig. 5

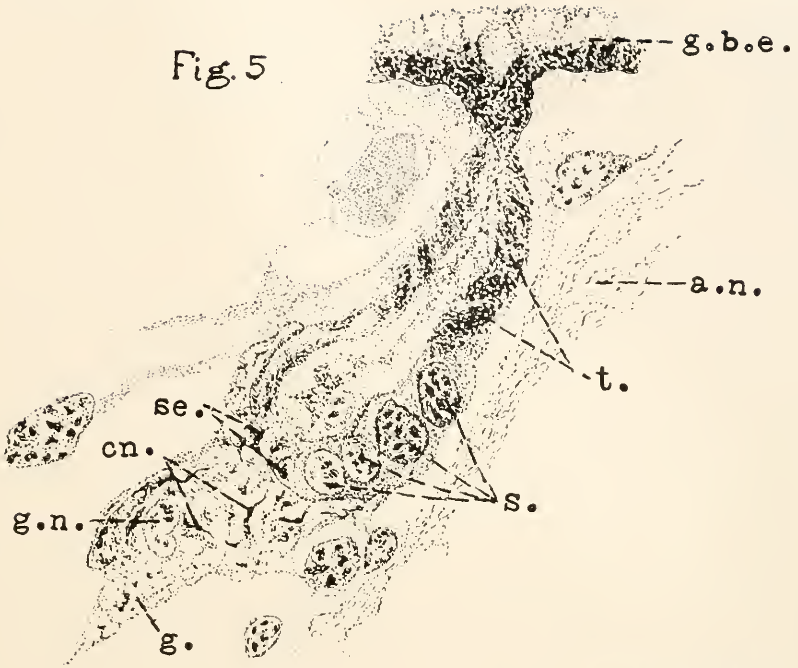
g.b.e.
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Fig. 4

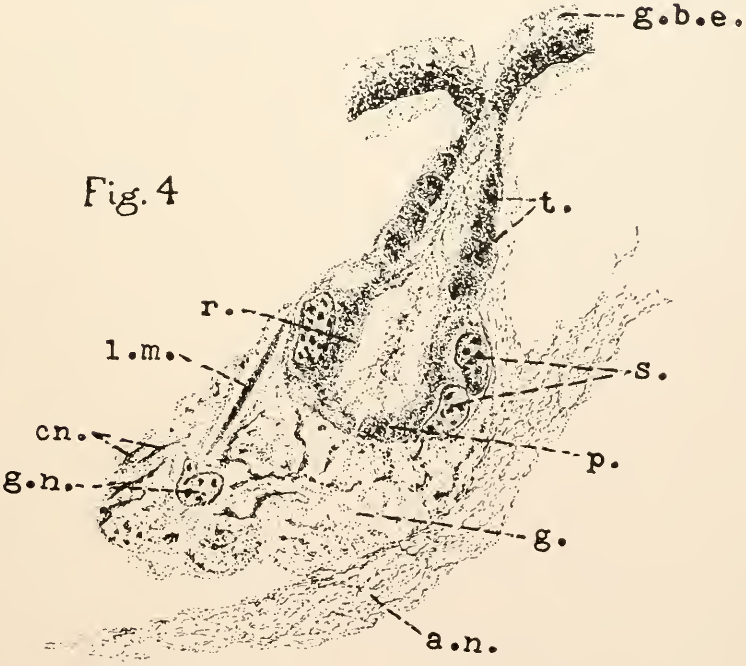
g.b.e.
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PLATE III.

FIG. 6. Oblique-longitudinal section through right ciliated pit especially to show the three mesial muscle fibers (*m.m.*); associated with the ciliated pits. Sensory nucleus (*s.*); transitory nucleus (*t.*); pores of gland cell (*p.*); canaliculi (*cn.*); gland cell (*g.*); anterior nerve (*a.n.*); non-ciliated ridge (*r.*); × 1800.

FIG. 7. Diagrammatic reconstruction of lateral aspect of right pit. General body epithelium (*g.b.e.*); transitory nuclei (*t.*); mesial application of anterior nerve (*a.n.*); sensory nucleus (*s.*); pores of gland cell (*p.*); emptying into lumen of pit; canaliculi (*cn.*); gland cell (*g.*); bent, ovate nucleus of gland cell (*g.n.*); lateral muscles of wall of pit (*l.m.*); mesial muscles of wall of pit (*m.m.*); × 2000.

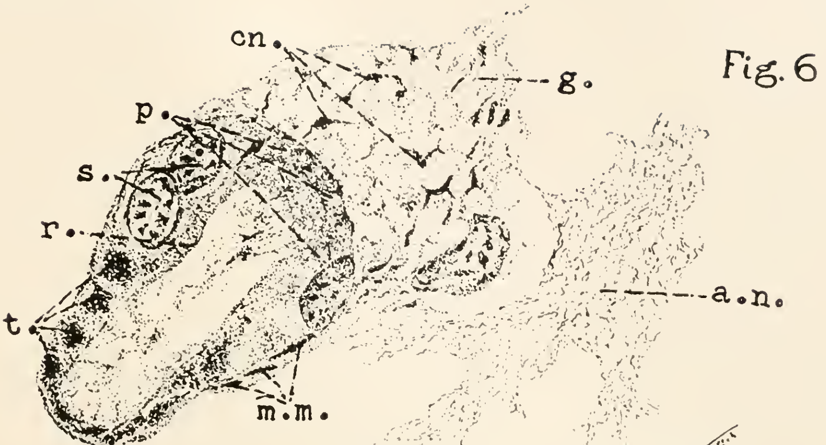

Fig. 6

cn.
g.
p.
s.
r.
t.
a.n.
m.m.

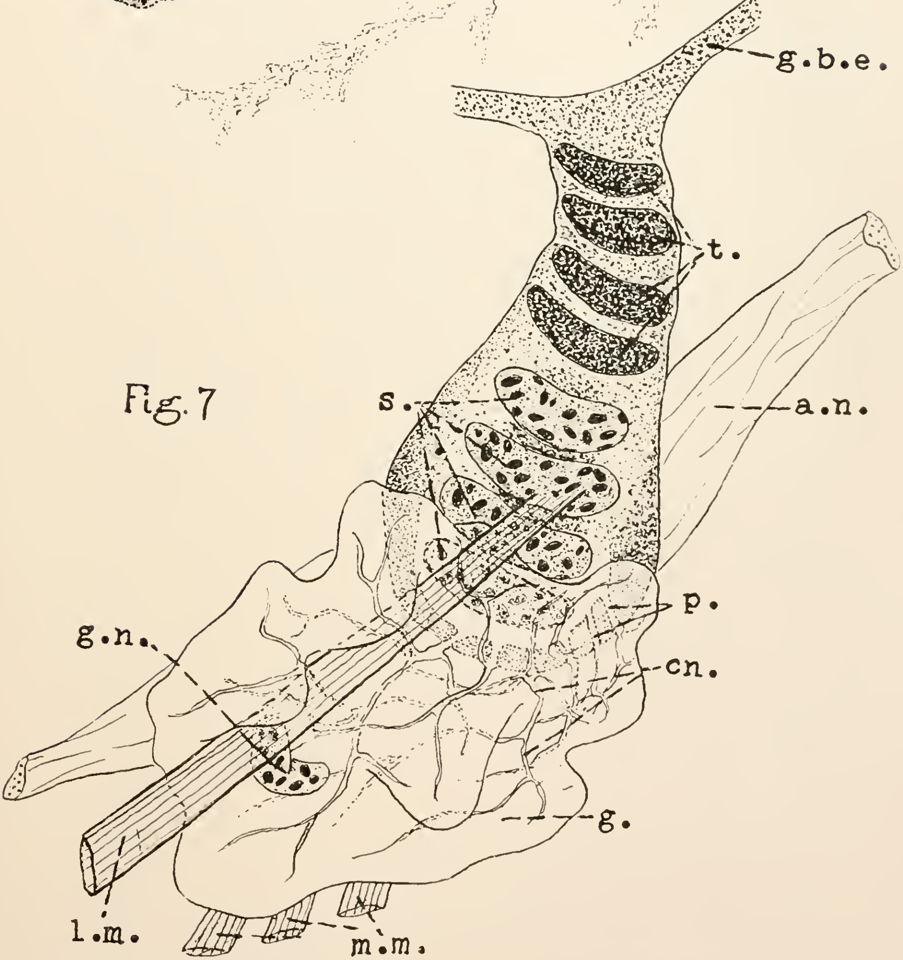

Fig. 7

g.b.e.
t.
s.
a.n.
p.
g.n.
cn.
g.
l.m.
m.m.

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TESTICULAR ASYMMETRY AND SEX RATIO IN BIRDS.

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It has long been known that in some birds the right testis never attains the size of the left one even at the height of the vernal hypertrophy of the gonads. Newton, in his Dictionary of Birds (p. 784) writes that, ". . . generally the left testis is bigger than the right, although both are equally functional." Riddle,[1] working with pigeons at Cold Spring Harbor, found that the discrepancy in size between the two testes in hybrids increased with the width of the cross involved. That is to say, the left testis was proportionately larger in hybrid birds whose parents were of two different genera than in birds resulting from the mating of two congeneric species. The sex ratio of the offspring was found to be apparently interrelated with the discrepancy in size of the testes. Excess of males was always correlated with proportionately larger left testes, while in birds in which the two testes were equal in size the sex ratio was approximately one, *i.e.*, as many of one sex as of the other.

If there is anything in this seeming correlation it should be possible to arrive at some idea of the sex ratio in wild species of birds by examining the testes of adult males in breeding condition. Those in which the left testis is much larger than the right should have an excess of males in their total population and this excess should vary directly with the amount of difference between the two testes of the adult male. The importance of the sex ratio in such matters as the courtship habits and territorial relations of birds must be very great and its accurate determination for any species is prerequisite to a proper understanding of the habits of that species.

With the hope of getting some new light on this subject in wild birds I kept note of the relative size of the testes of all adult male birds in breeding condition collected during three expeditions to South America, Africa, and the Texan-Mexican border. Only adult males in full breeding condition were of value in this study for in all the birds examined not in breeding condition the testes were invariably equal in size and very small.

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[1] Riddle, Oscar, "Further Observations on the Relative Size of the Right and Left Testis of Pigeons in Health and Disease and as Influenced by Hybridity," *The Anatomical Record*, XIV., 1918, pp. 333–4.

In no species was the right testis larger than the left. The two were equal in size in one hundred and four species and the left testis was larger than the right (usually by a very considerable amount) in sixty species. Five species showed both conditions. This is hardly in keeping with Newton's statement quoted above. It should be borne in mind that many of the species are included in the following lists on the basis of the condition found in a single adult male. With further data and the elimination of individual variation the status of some of the species may require modification in this respect.

From my own field notes on all these birds and from all available published accounts of other observers it seems that no general correlation exists between the relative size of the testes of the breeding male and the sex ratio of the species. That is, field observations do not indicate a larger proportion of males in those species exhibiting testicular size assymetry than in those in which the two testes are equal. However it must be admitted that sex ratio is a very difficult thing to determine in the field, particularly as male birds are usually more conspicuous than females and are therefore seen and collected more frequently. In some small groups of species there does seem to be some such correlation as Riddle found in his pigeons. Perhaps the most striking case (and one on which I have sufficient material to eliminate individual variation) is that presented by the Cowbirds.

The Cowbirds are a group comprising three genera and six species. One[2] of these six species is extremely rare, being represented in the museums of the world by only one adult and three immature specimens and I have no data on it. The condition in the other five is interesting and suggestive. Two of them, *Agelaioides badius* and *Molothrus rufo-axillaris*, have no sexual dimorphism in plumage, are monogamous, and the ratio of the sexes is even, one male to one female. These two species have the testes equal in size. The other three *Molothrus bonariensis*, *Molothrus ater*, and *Tangavius æneus*, have sexual dimorphism in plumage, are more or less promiscuous with a tendency towards polyandry, and the males in all three outnumber the females by at least three to two, or by not less than fifty per cent. In these three species the left testis is much larger than the right. It is interesting to note that of the five the first two (with equal testes in breeding adult males) are the most primitive species of Cowbirds in all respects. *Agelaioides badius* is more or less normal in its reproductive habits but all the others are parasitic, *i.e.*, have no nests of their own and lay their eggs in nests of other birds and leave them to be cared for by these foster-parents. The development of this habit allowed for an increase of males in proportion to females as true mating and pairing were probably of less importance to a parasitic species than to one tied down by nesting and parental obligations. The parasitic habit is simplest in *Molothrus rufoaxillaris* and this species still pairs off in its ancestral monogamous fashion as does the most primitive, non-parasitic *Agelaioides badius*.

---

[2] *Tangavius armenti.*

In most birds studied however no correlation between testicular asymmetry and sex ratio can be made out. Many more data are needed and I hope that bird collectors will make a point of noting the relative size of the testes in all breeding adult males.

Birds in which the Two Testes are Equal in Breeding Adult Males.

Family Ciconiidæ.

Euxenura galatea (Molina).

Family Tinamidæ.

Nothura maculosa maculosa (Temminck).

Family Rallidæ.

Gallinula chloropus brachyptera (Brehm).

Family Charadriidæ.

Charadrius pecuarius pecuarius Temminck.

Family Jacanidæ.

Jacana jacana (Linneus).

Family Laridæ.

Sterna maxima Boddært.
Geochelidon nilotica (Gmelin).

Family Cuculidæ.

Crotophaga ani Linneus.
Guira guira (Gmelin).
Tapera nævia chochi (Vieillot).
Lampromorpha klaasi (Stephens).
Clamator cafer (Lichtenstein).
Coccyzus melocoryphus (Vieillot).
Coccyzus americanus americanus (Linneus).
Coccyzus erythrophthalmus (Wilson).

Family Coraciidæ.

Coracias caudatus caudatus Linneus.

Family Bucerotidæ.

Lophoceros erythrorhynchus erythrorhynchus (Temminck).

Family Trogonidæ.

Apaloderma narina narina (Stephens).

Family Trochilidæ.

Chlorostilbon aureoventris egregius Heine.
Heliomaster furcifer (Shaw).

Family Capitonidæ.

Trachyphonus erythrocephala erythrocephala Cabanis.

Family Indicatoridæ.

Indicator indicator Gmelin.
Indicator minor teitensis Neumann.

Family Picidæ.

Picumnus cirrhatus pilcomayensis Hargitt.
Campethera nubica pallida (Sharpe).
Trichopicus cactorum (d'Orbigny).

Family Furnariidæ.

Coryphistera alaudina alaudina Burmeister.
Phacellodomus rufifrons sincipitalis Cabanis.
Synallaxis superciliosa Cabanis.
Synallaxis albescens albescens Temminck.

Family Tyrannidæ.

Tænioptera irupero (Vieillot).
Fluvicola albiventer (Spix).
Euscarthmornis margaritaceiventer margaritaceiventer (d'Orbigny et Lafresnaye).
Elænia albiceps albiceps (d'Orbigny et Lafresnaye).
Empidagra suiriri (Vieillot).
Myiodynastes solitarius (Vieillot).
Pitangus sulphuratus bolivianus (Lafresnaye).
Tyrannus melancholicus melancholicus Vieillot.
Muscivora tyrannus (Linneus).

Family Alaudidæ.

Mirafra sabota Smith.
Mirafra africana transvaalensis (Hartert).
Eremopteryx leucopareia Fischer et Reichenow.

Family Motacillidæ.

Macronyx aurantiigula Reichenow.
Macronyx capensis (Linneus).
Anthus cafer (Sundeval).
Anthus leucophrys (Vieillot).

Family Timeliidæ.

Argya rubiginosa saturata Sharpe.

Family Pycnonotidæ.

Pycnonotus tricolor minor Heuglin.

Family Muscicapidæ.

Batis capensis (Linneus).
Alseonax adustus adustus (Boie).
Melænornis ater pammelaina (Stanley).
Elminia longicauda teresita Antinori.

Family Turdidæ.

Planesticus amaurochalinus (Cabanis).

Family Sylviidæ.

Erythropygia pæna pæna (A. Smith).
Sylvietta whytii loringi Mearns.
Cisticola semifasciata Reichenow.
Cisticola chiniana (A. Smith).
Polioptila cærulea cærulea (Linneus).
Polioptila dumicola (Vieillot).

Family Troglodytidæ.

Troglodytes musculus rex (Berlepsch et Leverkuhn).

Family Dicruridæ.

Dicrurus adsimilis adsimilis (Bechst.).

Family Laniidæ.

Eurocephalus rueppelli erlangeri Zedlitz.

Family Prionopidæ.

Prionops poliocephala talacoma A. Smith.

Family Vireonidæ.

Vireo chivi chivi (Vieillot).
Vireosylva olivacea (Linneus).

Family Paridæ.

Anthoscopus musculus (Hartlaub).

Family Oriolidæ.

Oriolus larvatus larvatus Lichtenstein.

Family Sturnidæ.

Amydrus morio morio (Linneus).
Spreo superbus (Ruppell).

Family Eulabetidæ.

Cinnyricinclus verreauxi (Finsch et Hartlaub).
Lamprocolius phœnicopterus bispecularis (Stickland).

Family Buphagidæ.

Buphagus erythrorhynchus (Stanley).

Family Zosteropidæ.

Zosterops virens Sundeval.

Family Nectariniidæ.

Nectarinia nectarinoides (Richmond).

Family Compsothlypidæ.

Dendroica æstiva æstiva (Gmelin).
Geothlypis æquinoctialis cucullata (Latham).
Setophaga ruticilla (Linneus).

Family Tanagridæ.

Thraupis bonariensis (Gmelin).

Family Ploceidæ.

Hyphantornis capensis olivaceus (Hahn).
Hyphantornis rubiginosus (Ruppell).
Hyphantornis nigriceps nigriceps Layard.
Hyphantornis jacksoni (Shelley).
Hyphantornis vitellinus uluensis Neumann.
Sycobrotus gregalis (Lichtenstein).
Euplectes xanthomelas Ruppell.
Pyromelana diademata (Finsch et Reichenow).
Pytelia melba melba (Linneus).
Granatina granatina (Linneus).
Sporopipes squamifrons squamifrons (A. Smith).
Dinemellia dinemelli (Ruppell).
Philetairus socius socius (Latham).
Tetrænura regia (Linneus).

Family Bubalornithidæ.

Bubalornis niger intermedius (Cabanis).

Family Icteridæ.

Agelaioides badius badius (Vieillot).
Molothrus rufo-axillaris Cassin.
Icterus cucullatus sennetti Ridgway.

Family Fringillidæ.

Embernagra olivacens olivascens (d'Orbigny et Lafresnaye).
Saltator cærulescens cærulescens Vieillot.
Saltatricula multicolor (Burmeister).
Serinus mozambicus mozambicus (Linneus).
Sicalis pelzelni Sclater.
Sporophila lineola (Linneus).
Brachyspiza capensis argentina Todd.
Melospiza melodia melodia (Wilson).

BIRDS IN WHICH THE LEFT TESTIS IS LARGER THAN THE RIGHT.

Family Ardeidæ.

Butorides striatus cyanurus (Vieillot).

Family Palamedeidæ.

Chauna torquata (Oken).

Family Phasianidæ.

Francolinus coqui coqui (Smith).

Family Rallidæ.

Limnocorax flavirostra (Swainson).

Family Charadriidæ.

Charadrius collaris Vieillot.
Hoplopterus armatus (Burch.).

Family Laridæ.

Sterna superciliaris Vieillot.

Family Cuculidæ.

Cuculus clamosus jacksoni Sharpe.
Cuculus solitarius Stephens.
Clamator serratus (Sparrmann).
Chrysococcyx cupreus intermedius Hartlaub.
Centropus superciliosus furvus Friedmann.

Family Alcedinidæ.

Halcyon chelicuti chelicuti (Stanley).
Halcyon albiventris albiventris (Scop.).

Family Caprimulgidæ.

Setopagis parvulus (Gould).

Family Coliidæ.

Colius striatus minor Cabanis.

Family Indicatoridæ.

Prodotiscus regulus regulus Sundeval.

Family Phœniculidæ.

Phœniculus purpureus marwitzi (Reichenow).

Family Bucconidæ.

Nystalus maculatus striatipectus (Sclater).

Family Furnariidæ.

Furnarius rufus rufus (Gmelin).

Family Tyrannidæ.

Machetornis rixosa rixosa (Vieillot).
Pyrocephalus rubineus rubineus (Boddært).
Knipolegus aterrimus aterrimus Kaup.

Family Phytotomidæ.

Phytotoma rutila rutila Vieillot.

Family Alaudidæ.

Mirafra fischeri fischeri Reichenow.

Family Muscicapidæ.

Tchitrea viridis perspicillata Swainson.
Lioptilus nigricapillus (Vieillot).

Family Mimidæ.

Mimus saturninus modulator (Gould).

Family Campephagidæ.

Graucalus cæsia cæsia (Lichtenstein).

Family Laniidæ.

Chlorophoneus sulphureopectus suahelicus Neumann.
Laniarius funebris degener Hilgert.

Family Nectariniidæ.

Cinnyris mariquensis mariquensis Smith.
Cinnyris obscura neglecta Neumann.
Cinnyris chalybeus (Linneus).
Cinnyris amethystina amethystina (Shaw).

Family Ploceidæ.

Hyphantornis spilonotus (Vig.).
Melanopteryx nigerrima (Vieillot).
Nigrita schistacea Sharpe.
Anaplectes rubriceps (Sundeval).
Anomalospiza imberbis Cabanis.
Coliuspasser ardens (Boddært).
Coliuspasser eques Hartlaub.
Coliuspasser albonotatus (Cassin).
Diatropura procne (Boddært).
Urobrachya axillaris (A. Smith).
Pyromelana orix orix (Linneus).
Linura fischeri (Reichenow).

Family Icteridæ.

Agelaius phœniceus phœniceus (Linneus).
Molothrus ater ater (Boddært).
Molothrus ater obscurus (Gmelin).
Molothrus bonariensis bonariensis (Gmelin).
Tangavius æneus involucratus Lesson.
Quiscalus quiscula æneus (Ridgway).
Leistes superciliaris petilus Bangs.

Family Fringillidæ.

Passer domesticus (Linneus).
Passer melanurus (P. L. S. Muller).
Serinus canicollis thompsonæ Roberts.
Poliospiza gularis gularis (A. Smith).
Paroaria cristata (Boddært).
Emberiza flaviventris Stephens.

Variable Species.

Family Cuculidæ.

Cuculus clamosus clamosus Latham.
Lampromorpha caprius (Boddært).

Family Dendrocolaptidæ.

Lepidocolaptes angustirostris angustirostris (Vieillot).

Family Ploceidæ.

Vidua macroura (Vroeg.).
Hypochera orientalis Reichenow.

THE FUNCTION OF THE CONTRACTILE VACUOLE IN *PARAMECIUM CAUDATUM;* WITH SPECIAL REFERENCE TO THE EXCRETION OF NITROGENOUS COMPOUNDS.[1]

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Introduction.

Ehrenberg (1838) was probably the first to consider the function of the contractile vacuole. He asserted that it is a spermatic gland; but no evidence has been found in support of this view.

Lieberkühn (1836), Claparède (1834), Lachmann (1856), Siebold and Stannius (1854), and Pritchard (1861) held that it is a rudimentary heart, which, by its pulsations, produces the circulation of a body-fluid throughout the organism. The demonstration by Jennings (1904) that the vacuole communicates directly with the exterior and discharges its contents into the surrounding medium definitely eliminates such an explanation.

Haeckel (see Kent, 1880, p. 69), Maupas (1863), Bütschli (1887–89), Ehrmann (1894), and others contend that it is a respiratory organelle, or a mechanism for the removal of some of the end products of oxidation; but insufficient evidence has been found to warrant the acceptance of this view.

Stein and Schmidt (see Kent, 1880, p. 69), Griffiths (1888), Calkins (1909), Khainsky (1910), Woodruff (1911), Minchin (1912), Howland (1924), Nowikoff (1908), Shumway (1917), Riddle and Torrey (1923), Flather (1919), and Marshall (1921) believed the vacuole to be an excretory organelle. This view is widely accepted, and is supported to some extent by experimental evidence. In certain instances, the term excretion is limited to mean only the expulsion of a fluid from the cell, as in Carter's (1861) observations. Generally, however, this theory assigns to the vacuole the function of a renal organ of some kind.

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[1] This paper is a portion of an essay submitted to the Board of University Studies of the Johns Hopkins University in partial fulfillment of the requirements for the degree of Master of Arts. The writer wishes to acknowledge his indebtedness to Professor S. O. Mast, who suggested the problem, and under whose personal supervision the work was done; and to Professor E. K. Marshall of the Department of Physiology of the Johns Hopkins Medical School who offered many helpful suggestions in connection with the literature on the enzyme, urease, and the xanthydrol test for urea.

Hartog (1888), Calkins (1901), Zülzer (1910), Doflein (1911), and others maintain that the vacuole is an organelle for regulating the hydrostatic pressure within the cell, or a mechanism for removing the excess water which is taken into the body in feeding and through the cell membrane by osmosis. Stempell (1914) constructed a mechanical system which shows clearly that osmosis can be made the causal agent for producing intermittent discharge of a fluid from such a system, but this system was doubtless not intended to be compared with the vacuole in any respect other than the pulsating effect.

The literature concerned with the function of the contractile vacuole consequently reveals no conclusive evidence in support of one theory to the exclusion of all others. The consensus of the evidence, however, seems to indicate that the vacuole is an organelle concerned either with the removal of waste products of metabolism, or with the removal of excess water which accumulates in the organism as a result of endosmosis and feeding. The experiments described in the following pages have a direct bearing on the question as to which of these obtains. These experiments consist of attempts to ascertain the nature of the nitrogenous excretion products of metabolism in *Paramecium caudatum*, and whether or not they are excreted through the contractile vacuole.

The Nature of the Nitrogenous End Products in *Paramecium*.

The nitrogenous end products of metabolism in organisms vary in their nature according to the type of organism. For example, in man the bulk of the nitrogen is eliminated as urea, considerably less as uric acid and amino acids, and a very small part as free ammonia; while in the birds and reptiles the bulk of the nitrogen is eliminated as uric acid.

In connection with protozoa Griffiths (1888) made the statement, based on his own experiments, that the vacuole performs the function of a kidney, and that its secretions are "capable of yielding microscopic crystals of uric acid." As material for these experiments he used Amœba, Paramecium, and Vorticella, in mass cultures. In some of the experiments a number of amœbæ were placed on a slide and subjected to the murexide test. The development of reddish-purple color indicated the presence of uric acid. In describing these experiments Griffiths says (p. 132): "After the addition of alcohol minute flakes could be distinctly seen floating in the fluid of certain vacuoles. Bearing in mind the murexide reaction, there is every reason to believe that these flakes are nothing more or less than minute crystals of uric acid." These experiments were repeated many times, generally with positive results, indicating the presence of uric acid. At times, however, the vacuole was found not to contain the slightest trace of uric acid.

Howland (1924) repeated these experiments using several specimens of *Centropryxis* and *Amœba verrucosa.* Cells observed in a dark field immediately after the addition of alcohol did not show the crystals of uric acid in the distended vacuoles, nor did cells after the addition of ammonia show the characteristic murexide reactions, either in the vacuoles or in the culture medium immediately surrounding the organisms. These experiments were conducted during a period of five weeks, always with negative results. Paramecia were subjected to the same test, also with negative results. Howland made use of the Benedict blood-filtrate test for uric acid on cultures of paramecia and amœbæ with positive results. The depth of the color developed varied with the age of the cultures, the older ones giving a deeper color. This indicates that uric acid was eliminated in some way by the organisms.

The question then arises: Is nitrogen excreted by protozoa as ammonia, urea, uric acid, or a combination of these substances? In an effort to answer this question the following experiment was conducted. A large number of paramecia were thoroughly washed, placed in spring water that was free from ammonia and urea, left for a time, and removed by filtration. The filtrate was then tested for ammonia and urea as described below. The possible presence of uric acid was not investigated at this time.

The paramecia were washed as follows: Culture fluid containing the organisms was poured into a long-necked bottle of approximately one liter capacity until it was filled to within about 5 cm. of the top. Then spring-water was added carefully so as to avoid mixing until the bottle was entirely full. Within five or ten minutes under these conditions the paramecia usually aggregated in very great numbers near the surface of the spring-water. When they had thus collected at the top of the bottle, the surface water containing them was removed with a pipette, and more spring-water added. This process was repeated until most of the animals had been taken from the culture fluid, usually three or four times. The bottle was then emptied, and the water containing the paramecia put into it, after which it was filled with spring-water. The organisms were removed as before. The paramecia were thus washed in fresh spring-water three or four times, after which they were usually found to be free from all heavy debris and large bacterial masses. Smaller organisms which were removed from the bottle with the paramecia were separated from the paramecia by further washing on filter paper. The paper used was about 20 cm. in diameter, and was selected so that the pores were small enough to retain the paramecia, but large enough to allow the smaller organisms to pass through. A liter of water, or more if necessary, was used for this part of the washing process.

After the paramecia had been thus washed they were put into a clean glass beaker; then spring-water was added until the number per cubic centimeter was reduced to from 500 to 2,500 individuals. This was ascertained by counting the numbers in several one cubic centimeter portions and averaging the results obtained. The paramecia were allowed to remain in this water for periods of time ranging from eighteen to thirty-six hours, after which they were removed by filtration.

A portion of the filtrate was tested for ammonia by Nesslerization and the rest for urea by methods described below.

In twenty-two of the twenty-five experiments positive tests were obtained for ammonia by Nesslerization. In the three in which no ammonia was found the paramecia had been in the water for a period of thirty hours or less. In other experiments in which the length of time was thirty-six hours or more, ammonia was invariably found. This indicates that either ammonia was eliminated in such small amounts that more than thirty hours were required for the concentration to rise sufficiently high to be detected by Nesslerization; or that no ammonia was eliminated as such, the positive test being due to that formed from the hydrolysis of some other excretion product. The latter seems the more probable, for, if ammonia was excreted, the length of time necessary for its concentration to rise sufficiently high to be detected should bear an inverse relation to the number of paramecia per unit volume of water. No relation of this kind was found to exist. The ammonia appeared after thirty to thirty-six hours in all the experiments regardless of the number of animals present. The maximum variation, then, in the length of time necessary for ammonia to make its appearance was twenty per cent., while the variation in the number of paramecia in these same experiments was one hundred per cent. or more. The absence of ammonia in three experiments, and its presence in all the others can be explained if it is assumed that there were too few bacteria present in the three to produce hydrolysis of the more complex excretion products, while in the other experiments there were enough bacteria present. That ammonia can be produced in this way was demonstrated by inoculating a dilute solution of urea with culture fluid. After the solution had been allowed to stand for several hours it gave a positive test for ammonia with Nessler's reagent, indicating that hydrolysis had taken place.

Many tests were made of the materials used in these experiments to prevent the possible introduction of errors. The spring-water was tested for ammonia. The sensitivity of Nessler's reagent was ascertained by finding the greatest dilution possible at which a definite indication of ammonia could be obtained. This dilution was found to be approximately one part in two million. The filter paper on which the paramecia were washed was tested for ammonia.

To ascertain whether or not ammonia is present in the fluid of the vacuole, fifteen experiments were conducted in which Nessler's reagent was injected into the organism. The apparatus used in making these injections consisted of the micropipette developed by Taylor (1925) mounted on the micromanipulator developed by Chambers (1922). The process of injection was performed with the paramecium held by surface tension in a hanging drop of water. The cover-glass bearing the organism formed the top of a cell, the front of which was left open to allow the micropipette entrance. The tip of the pipette was bent up at a right angle to the main shaft to facilitate the injection of the organism suspended on the lower surface of the cover-glass.

In twelve of the injections the contents of the pipette were discharged into the vacuole. In three the pipette did not penetrate the vacuole, but discharged its contents into the cytoplasm in the immediate vicinity of the vacuole. In every test the reagent, which is highly caustic, caused the immediate solution of the whole organism with the exception of the nucleus, which remained intact for a short time before it too was dissolved. In the three tests in which the pipette did not penetrate the vacuole, the surrounding cytoplasm was dissolved as before, but the membrane around the vacuole remained intact for a short time. After several seconds the membrane was dissolved, causing the contents of the vacuole to be emptied into the solution of Nessler's reagent in which it was floating. In none of these tests was the characteristic straw color observed which, in the presence of Nessler's reagent, indicates ammonia. It seems, then, that if ammonia is present in any part of the organism its concentration is below the sensitivity of the reagent. All of these experiments seem to indicate then that very little if any of the nitrogen found in the excretion products of *Paramecium* is excreted in the form of ammonia.

The test for urea referred to above was made as follows: Urease, a specific enzyme for urea, hydrolyzing it into ammonia and carbon dioxide, was added to the portion of the filtrate not used for the test for ammonia in each of the twenty-five experiments mentioned. They were then left for several hours, after which they were tested for ammonia by Nesslerization. Ammonia was found in the filtrate from every experiment, and generally in higher concentrations than it was in the portions to which no urease had been added. This increased ammonia content after hydrolysis may, then, with a reasonable degree of certainty, be interpreted as indicating that urea from some source had been hydrolyzed with the subsequent production of ammonia. The fact that the three filtrates which gave no indication of ammonia before hydrolysis, gave a distinctly positive test for ammonia after hydrolysis, is alone conclusive in so far as the action of urease is known to be limited to the hydrolysis of urea. Since paramecia were the only organisms present in the water in any considerable numbers, the source of this urea must be attributed to them. It therefore seems evident that in *Paramecium* at least some of the nitrogen is excreted in the form of urea.

Is Urea Eliminated by the Contractile Vacuole?

In an effort to answer this question the xanthydrol precipitation test for urea, described by Fosse (1913) and modified to suit conditions of this experiment, was made by injecting the reagent into the vacuoles. The modified reagent consisted of three to five drops of a ten per cent. solution of xanthydrol in methyl alcohol, in 1 cc. glacial acetic acid. This reagent, in the presence of urea, precipitates long, needle-like crystals of di-xanthyl urea which may be easily recognized. The sensitivity of the modified reagent was found by injecting it, with the aid of the apparatus described above, into a drop of a solution of urea of known concentration. The solution of urea used was successively diluted until the urea content was so low that no precipitate could be observed. It was found that one or more parts of urea in twelve thousand could be detected. The process of injecting the reagent into the drop of solution was observed under a microscope.

Considerable annoyance was encountered in attempting to inject the contractile vacuole of *Paramecium* with the xanthydrol reagent in that fumes from the acetic acid in the pipette killed the organism before the injection could be made. This difficulty was finally overcome by drawing into the pipette a very small amount of paraffine oil after the pipette had been filled with the reagent. The oil is chemically inert under ordinary conditions and served the purpose very well.

The effect of the reagent on the five paramecia successfully injected was quite striking. The animal was fixed immediately. It assumed an almost hyaline appearance with the exception of the nucleus, food granules, and numerous short, thick crystals which are normally found throughout the body. The contractile vacuole disappeared completely. No trace of the characteristic needle-like crystals of di-xanthyl urea, which are precipitated by xanthydrol in the presence of urea, were found either in that part of the organism in which the vacuole is usually situated, or in the liquid surrounding the organism. Some of these observations were made under an apochromatic oil immersion lens system. It seems from this, then, that if urea is present in the fluid of the vacuole its concentration is too low to be detected with the reagent used, that is, one part in 12,000.

Now the question arises as to whether or not all of the urea excreted could be eliminated by the contractile vacuole if the concentration is as low as this. If not, then it is evident that the contractile vacuole does not function specifically in the excretion of nitrogen, and if this is true it is not an excretory organelle in the ordinary sense of the term.

The concentration of urea that should be in the fluid of the vacuole, if all of it is eliminated through it, was ascertained in the following manner. Maupas (1883) found that the vacuoles of *Paramecium aurelia* evacuate a quantity of water equal to the volume of the entire organism in forty-six minutes at twenty-seven degrees. It was assumed in making these calculations that the relative quantity of water evacuated by *Paramecium caudatum* is approximately equal to that evacuated by *Paramecium aurelia* during the same period and at the same temperature. The average volume of *Paramecium caudatum* was assumed to be that of a cylinder 150 microns long and 35 microns in diameter, and the diameter of the vacuole when distended 10 microns.

On the basis of these assumptions and the observations of

Maupas, the volume of water evacuated by a definite number of paramecia in a definite period of time was calculated. From this the concentration of urea that would be in the fluid of the vacuole, if its function is excretory, was computed. The results of these calculations show that the concentration of urea in the fluid of the vacuole would have to be of the order of one part in two or three thousand to eliminate through it the calculated amount of urea.

The reagent injected into the vacuole is, as previously stated, sensitive to one part of urea in twelve thousand. Since there was in these injections no indication of the presence of urea, it is evident that the results of these experiments are in opposition to the theory that the vacuole is an organelle whose function is the removal of the nitrogenous waste products of metabolism, unless it functions specifically in eliminating uric acid, which is not probable. If, then, it is true that the contractile vacuole functions either as an excretory organelle or a mechanism for regulating the hydrostatic pressure within the cell, it is evident that the results support the latter.

Summary

1. The presence of ammonia and urea in *Paramecium* cultures has been demonstrated.

2. Ammonia is due to the hydrolysis of urea, and is not excreted as such.

3. Nitrogen is eliminated in the form of urea.

4. All the urea excreted can not be eliminated through the vacuole.

5. The function of the vacuole is not the elimination of nitrogenous waste products of metabolism, but is probably the regulation of the hydrostatic pressure within the cell.

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THE USE OF NILE BLUE SULFATE AS A VITAL STAIN ON *HYDRA*.

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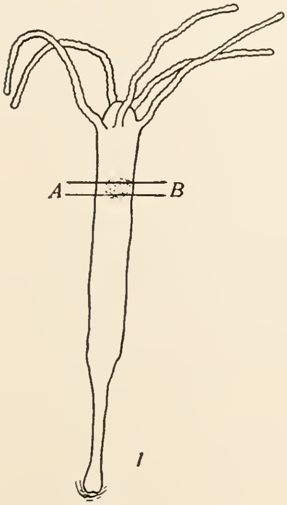
In connection with some studies on the reconstitution of *Hydra*, it occurred to the writer that if some method could be devised for marking the various regions in an animal and if these markings would persist, the process of reconstitution could be followed and interpreted much more easily and accurately. However, in reviewing the literature there seems to be little or no application of any of the so-called vital dyes to the invertebrates, particularly the Cœlenterata.

Various investigators in studying regeneration in *Hydra* following grafting have made use of animals of two different species which differed from each other, among other characteristics, in color. In this way the fate of the graft could be traced. Others have used individuals of the same species which differed from each other in color shade. Both of these procedures are open to criticism in interpretation of results. In the case first mentioned there are undoubtedly present species differences other than color between the animals used, such as differences in the metabolic rate of the animal as an individual and differences in gradient patterns. In the second case in individuals of the same species, color differences seem to be indicative of different physiological conditions. Observations tend to show, for example, that young *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.) are lighter in color than old animals. The same color difference seems to be true of starved animals as compared to fed. From data gathered by various investigators on *Tubularia* and *Planaria*, there are marked physiological differences between young and old, and starved and fed animals with respect to metabolic rate and, consequently, capacity for reconstitution under various conditions. Obviously some method of vital staining would remove the necessity for using such methods of marking as previously described and permit experimentation under more normal, controlled conditions.

Of the various stains considered, nile blue sulfate seemed the most suitable for the work on *Hydra*, particularly on *Pelmatohydra* which is brown in color. The use of nile blue sulfate as a vital stain has been confined for the most part to embryological investigations and applied here for marking the eggs or the embryos. Goodale ('17) used nile blue sulfate in marking the eggs of *Spelerpes bilineatus*, the dye being applied locally in solid form in the least possible amounts by means of a needle. The dye particle was left for a few moments and then removed by washing. A too prolonged application or the application of the dye in excess amount was found to be toxic. Detweiler ('17) used the dye in aqueous solutions of 1 : 100,000 up to 1 : 500,000 but concluded that the ratio of 1 : 150,000 was optimum. In this case the entire animal was stained. Smith ('14) applied an aqueous solution by means of a fine pipette and was able to get some blue spots which persisted. Vogt ('25) uses nile blue sulfate in vital staining by first staining finely divided agar in aqueous solution of the dye (conc. 1 : 100 to 1 : 1,000) and after a few days applying in water one of the colored pieces of agar to the tissue to be stained. The color diffuses over after an interval of several hours to one day. However, the stain is not well localized. To localize the dye by this method, the stained agar must be inclosed in glass, tinfoil or paraffin.

After various trials, the method which was found most successful was that of Goodale. The animal, *Pelmatohydra oligactis* (Schulze) Pallas (*Hydra fusca* L.), was placed on a glass slide and the excess water removed from around the animal by means of filter paper. The animal was then transferred on the slide to a dissecting microscope and the smallest possible particle of nile blue sulfate applied in the desired region by means of a needle. Almost immediately the animal was washed from the slide into a stender dish containing water and thoroughly washed in currents of water by means of a pipette. A blue spot was found on the animal at the place of application of the dye. The animal was then sectioned in any way desired and in the following process of reconstitution of the pieces the blue color was found confined to the original colored cells or the new cells derived from this group as the following example will serve to illustrate.

An animal was stained as described above and a piece removed by two parallel cuts, transverse to the longitudinal axis of the animal (Fig. 1, *ab*). This cross-section, when removed from the animal, appeared as a ring of tissue with the blue spot (stippled area) at one point in the circumference (Fig. 2). This ring of tissue was then divided into two equal portions by a cut (Fig. 2, *cd*) which passed through the blue spot. The two pieces were allowed to reconstitute and at the end of four days two small animals were found similar in marking to Fig. 3.



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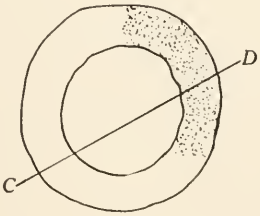

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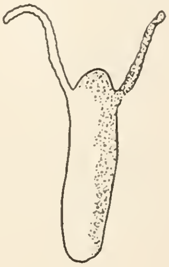

3

The process of reconstitution was as follows: the two cut lateral ends of each semicircle had approached and fused, thus forming two small rings similar to that seen in Fig. 2. Further process of reconstitution had then ensued governed by the original polarity of the pieces as is indicated by the color pattern as seen in Fig. 3. Further application of this staining method has been made in investigations not published as yet.

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BIOLOGICAL BULLETIN

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A LIPO-GEL REACTION EXERTED BY FOLLICULAR FLUID UPON SPERMATOZOA AND ITS SIGNIFICANCE (LILLIE'S REACTION).

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I. Introduction.

Some time ago Professor F. R. Lillie observed that when sperm suspensions are mixed with follicular fluid taken from the Graafian follicles a coagulum is obtained (unpublished). As this phenomenon may be significant for the process of fertilization, Professor Lillie suggested that I study the reaction and determine the exact conditions under which it appears. I desire to express my thanks to Professor Lillie for suggesting this subject of research and for placing at my disposal the facilities of the zoölogical laboratories of the University of Chicago.

II. Materials and Methods.

Several species were used in the study, chiefly cattle and sheep: Fresh organs obtained at the slaughter-houses of Swift and Company, Chicago,[2] were always employed. For comparison material was also taken from the goat, opossum, pig, and guinea-pig. The testes and ovaries were usually taken from the bodies of animals just killed and carried to the laboratory in a thermos bottle. Under these conditions the organs remained at a temperature of 25°–30° C. until they were used. The experiments were almost always completed within two to four hours after the removal of the organs from the animals. To establish certain points which required absolutely fresh material, the same series of experiments was performed twice, once at the slaughter-house, within a quarter of an hour after killing, and again in the laboratory.

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[1] From the Hull Zoölogical Laboratory and Whitman Laboratory for Experimental Zoölogy, University of Chicago.

[2] The author wishes to show his thanks to the officers of Swift and Co., who provided the material necessary for this work.

The spermatozoa were generally removed from different parts of the epididymis by transverse cuts through that structure. Sometimes they were also taken from the vas deferens and from the testes directly and also from the seminal vesicles. The follicular fluid was obtained by pricking the follicles at their most transparent points by means of a fine pipette into which the follicular fluid was drawn by suction.

All organs and especially the female genital tract were taken from the freshly killed animal by myself and examined immediately.

Other details of the technique will be described and explained in connection with the experiments.

III. Experiments.

1. When follicular fluid is mixed with spermatozoa taken directly from the epididymis or other parts of the male genital tract a coagulum is obtained in ten to twenty minutes. This coagulum is whitish, semi-opaque, friable, and of a specific gravity greater than that of water; when dissociated with needles, the coagulum breaks up into blocks with sharp edges, indicating a firm consistency. Small fragments of the coagulum when viewed under the microscope appear as homogeneous and transparent masses in which the spermatozoa are imbedded, scattered or in groups. After more complete dissociation of the coagulum into minute fragments one may isolate here and there bundles of fibers coated by a hyaline substance. The surface of the coagulum shines like a fatty surface and does not adhere to water. If therefore the coagulum is set carefully on the surface of water, it will float. When submerged, it sinks, showing that its specific gravity is greater than that of water.

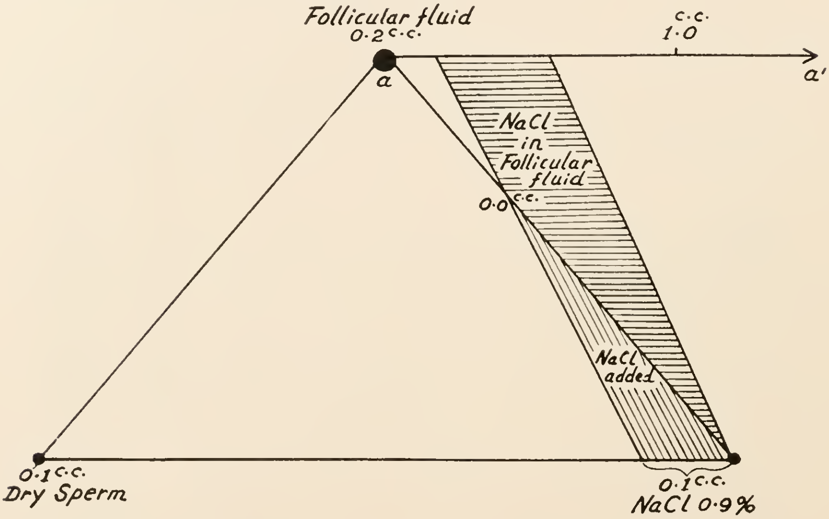
This coagulum is produced at all temperatures between 10° C. and 56° C.; higher temperatures are unfavorable for the reaction. After its formation the coagulum persists at temperatures to 100° C. The fluid around the coagulum produces a second coagulum when heated to 72° C.

2. When follicular fluid is mixed with spermatozoa only, the results are not uniform. For example in one series of 90 tests, the result was negative in 43 cases, positive in 36 cases, and uncertain in 10 cases. But if the spermatozoa are mixed with 0.9 per cent. sodium chloride solution the reaction is more certainly obtained. For instance in a series of 242 tests, 41 were negative, 198 positive, and 3 uncertain. Furthermore in many cases in which the reaction failed to appear, using follicular fluid and spermatozoa alone, the coagulum was produced upon the addition of 0.9 per cent. sodium chloride solution to the mixture.

This result suggested that the salts which are normally present in the follicular fluid play an important rôle in the reaction. The effect upon the reaction of several salts which commonly occur in organic liquids was thereupon tested. The following salts singly or in mixtures were tried: $NaCl$, KCl, $CaCl_2$, $MgCl_2$, $LiCl$, $NaHCO_3$, $KHCO_3$, and $CaCO_3$. All of these gave a negative result. Only sodium chloride is able to favor and accelerate the reaction.

Since, however, there were a number of negative cases even after the addition of sodium chloride, an explanation for the failure of the reaction in these cases was sought. It was found that the reaction always occurs when the constituents are employed in certain definite quantities. After numerous tests, the following proportions were found to be successful in nearly all cases: 0.9 per cent. sodium chloride, 0.1 cc.; follicular fluid, 0.2 cc.; dry sperm, 0.1 cc. In a series of 128 tests using these proportions only four tests were negative.

The rôle of these proportions in the reaction may be illustrated graphically as in Fig. 1. If too much sodium chloride is added to the same amount of sperm, then the dilution of the sperm is too great and the reaction fails to appear. On the other hand if the quantity of sperm is excessive, then the action of the salt solution upon the sperm—necessary for the reaction—is inadequate and the coagulum is not formed. With an excess of follicular fluid, the coagulum appears surrounded by a large quantity of fluid; if the quantity of follicular fluid is insufficient, the reaction fails also. From this we must conclude that some substance in the follicular fluid is essential for the formation of the coagulum. A minimum quantity of follicular fluid is thus necessary; this quantity (0.2 cc. to 0.1 cc. dry sperm) is indicated at the point *a* in the diagram. The line from *a* to *a′* indicates an indefinitely increasing quantity of follicular fluid in which the reaction is still possible, providing the proportions of dry sperm and sodium chloride solution remain constant in the ratio of 1 : 1. We must assume also an active maximum quantity for the sodium chloride solution, namely, a quantity equal to that of the amount of dry sperm. From this amount the proportion of sodium chloride may be decreased indefinitely to zero, at which the reaction is still possible, providing the follicular fluid contains sufficient sodium chloride.



The four negative cases obtained with correct proportions stated require explanation. The following possibilities are suggested:

(*a*) Possibly the essential substance in the follicular fluid was insufficient in quantity or lacking altogether.

(*b*) Possibly the quantity of sodium chloride normally present in the follicular fluid was lacking or reduced so that even after the addition of 0.1 cc. sodium chloride solution the amount of this salt present was still insufficient.

(*c*) Possibly the proportions of the three substances used was not perfectly correct as they were measured by drops usually.

For all practical purposes the proportions of follicular fluid, sperm and sodium chloride solution stated above are sufficiently accurate and seldom fail to yield the reaction.

3. After the conditions necessary for the production of the coagulum had been determined, a new series of experiments was undertaken to establish more definitely the nature of the reaction. The results may be summarized briefly.

(*a*) The reaction is not produced by other liquids of the organism. I added follicular fluid or dry sperm to the following body fluids: amniotic fluid, blood taken from the jugular vein, defibrinated blood, smear of the mucous membrane of the uterus and tube, urine, cerebrospinal fluid, aqueous humor of the eye, vitreous body of the eye, peritoneal fluid, and pericardial fluid. In all cases the result was negative.

(*b*) The reaction is interspecific. The following combinations were tried:

Dry Sperm.	Follicular Fluid.
ram	× cow
bull	× sheep
goat	× cow
goat	× sheep
opossum	× cow
guinea-pig	× cow
ram	× pig

In all of these tests the result was positive.

(*c*) Materials from different sources. Follicular fluid taken from one follicle of the cow was tested with sperm taken from the epididymes of ten different bulls. The result was positive in all cases. Follicular fluid from ten different ovaries of the cow was tried with dry sperm taken from the same epididymis of a bull with positive results in seven cases only. Probably some of the follicles had not reached the proper stage of concentration of the necessary constituents. However, follicular fluid taken from follicles of ten different sizes (cow and sheep), from the smallest to the largest gave a positive result in all cases. The age of the follicle does not appear to be of importance for the reaction.

(*d*) Time of reaction. In a series of ten tests (cow and sheep) observed after ten minutes, six were positive and four were negative. In ten tests observed after twenty minutes, all showed the coagulum. In a third series of ten tests, five of which were observed after one hour, five after 24 hours, all were positive. Thus the minimum average time necessary for the reaction lies between ten and twenty minutes. But the reaction is often observable after one minute. When dry sperm is mixed with follicular fluid on a slide, the reaction appears instantly. A delicate layer of coagulum is formed on the surface of the fluid.

(*e*) Variations in the time of addition of one of the components. In these experiments two of the three components were mixed and the third constituent added after a time interval, varying from one to twenty minutes. The following are the possible combinations:

(1) Follicular fluid mixed with salt solution; sperm added later.
(2) Sperm mixed with salt solution; follicular fluid added later.
(3) Sperm and follicular fluid mixed; salt solution added later.

No noticeable difference in the time of appearance of coagulum was noted in all three cases counting from the time of addition of the third constituent. In the third case the beneficial effect of salt solution on the reaction was again verified.

(*f*) Variations of temperature.

Dry sperm and follicular fluid were brought to various temperature and mixed (with the addition of salt solution as usual).

The results are summarized on opposite page.

These results show that there is in the follicular fluid a thermolabile substance which is responsible for the reaction. If the follicular fluid is heated to 56° C. the reaction fails. If the sperm is heated to a high temperature, even to 100° C. the reaction is obtained as long as follicular fluid is not heated above 55° C.

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Follicular Fluid.	Result.	Dry Sperm.
10° C.	Positive	10° C.
28° C.	“	28° C.
37° C.	“	37° C.
40° C.	“	56° C.
45° C.	“	57° C.
50° C.	“	56° C.
54° C.	“	56° C.
55° C.	“	56° C.
56° C.	Negative	56° C.
28° C.	Positive	56° C.
28° C.	“	72° C.
56° C.	Negative	100° C.
56° C.	“	28° C.

(*g*) Freshness of material. Dry sperm and ovaries were kept in the refrigerator and used for the test at various intervals. It was found that sperm fifteen days old and follicular fluid eight days old still yield a positive reaction either when tested with each other or with fresh sperm or follicular fluid respectively. The age of the materials therefore within the limits stated does not affect the reaction.

4. In order to obtain a better understanding of the nature of the reaction follicular fluid and dry sperm have been examined microscopically and the literature has been consulted as to the possible constituents present in these two fluids. I found that dry sperm may contain spermatozoa, spermatocytes, blood, and lipoid substances. Follicular fluid contains or may contain blood (red and white corpuscles and many substances in solution or suspension), granulosa cells, proteins, sodium chloride and other salts, and pigment. I attempted to determine which of these constituents are involved in the reaction.

(*a*) Blood. It is very difficult to avoid the admixture of blood into either sperm or follicular fluid owing to the cutting or pricking of blood-vessels in obtaining these substances. In my earlier experiments blood was constantly present in my tests and I therefore was inclined to ascribe the reaction to the presence of blood. Later, however, I took very minute precautions[1] to avoid admixture of blood and found that the reaction was always obtained in the complete absence of blood. I also while at the slaughter-house repeatedly mixed blood from the jugular vein with follicular fluid and never obtained the reaction.[2] It thus appears that the blood, and by implication the various constituents of the blood are not involved in the reaction.

(*b*) Proteins. A series of experiments performed with egg albumin gave completely negative results. This result would be expected from the negative finding with blood. Another proof that proteins are not involved may be derived from the fact that the reaction fails at a temperature of 56° C., although the fluid so heated and incapable of producing the reaction still contains proteins which do not coagulate until a temperature of 72° C. is reached.

(*c*) Sodium chloride. This salt and salts in general never produce the coagulum when mixed with either spermatozoa alone or follicular fluid alone.

(*d*) Granulosa cells. Follicular fluid was centrifuged and the clear fluid filtered to remove all cells. The absence of cells was verified by microscopic examination. The clear fluid was then violently agitated with glass granules for fifteen minutes with the idea of possibly altering the degree of dispersion of colloidal material that might be present. The fluid was then filtered through four sheets of soft filter paper. The fluid so treated still produces the reaction, indicating that cells or other formed constituents are not responsible for the coagulum. It may be mentioned here that filtered follicular fluid was always employed in all experiments.

(*e*) Spermatocytes. I was unable to separate the spermatocytes from the spermatozoa and therefore could not test their effect on the reaction separately. But I used material taken directly from the testes of animals of various ages and always obtained a positive reaction. Material from the testis may be supposed to contain a larger percentage of spermatocytes or cells in other stages of spermatogenesis than material taken directly from the epididymis or vas. No difference can be seen in the reaction however.

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[1] Ovaries taken directly from the body; washing them very carefully in laboratory before experiments; after that drying by absorbent paper; dissecting the follicles with a sharp knife until they appear like transparent vesicles. Only after that treatment puncture of follicles.

[2] In this case the anticoagulant effect of the sperm or of follicular fluid can be explained by the properties of NaCl and fatty substances, both able to stop the normal coagulation of the blood. Gilbert et Weinberg, "Traité du sang," 1913–1921, p. 15–16.

(*f*) Spermatozoa. Spermatozoa taken from any part of the epididymis, testis, vas deferens, or seminal vesicle always yield the reaction under the conditions previously described. Spermatozoa have been ground in a mortar for ten minutes, then mixed with salt solution, centrifuged, and filtered through four sheets of filter paper. The fluid obtained in this fashion was observed under the microscope; no spermatozoa or fragments of spermatozoa could be distinguished. Such a fluid however gives the reaction both with untreated follicular fluid and with follicular fluid that has been agitated, centrifuged, and filtered.

Thus all of the experiments to this point show that a coagulum is produced normally by a particular substance present in the follicular fluid, a product of the ovary, when mixed with products of the testes, especially the spermatozoa. The follicular substance is thermolabile and resistant to the action of mechanical factors. The experiments also show that the spermatozoa as formed cells are not essential to the reaction.

Up to this point in the experiments the reaction appeared to be perfectly specific: only products of the ovary act upon products of the testes.

5. Rôle of lipoids. Through certain microscopical observations, however, a new line of experimentation was suggested to me. Whenever spermatozoa treated with sodium chloride or with follicular fluid were studied under the microscope it was noted that after a short time the field of the microscope became filled with numerous small droplets, refractile, perfectly round, of the dimensions of cocci, and engaged in active brownian movements. The droplets increase in number with the degree of concentration of the salt solution.[1] When the suspension begins

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[1] This phenomenon of drop production by action of salt solution is very clearly explained in Aschoff, L., "Zur frage der tropfigen Entmischung," *Verh. deutsch. path. Gesell.*, 17, Tagung München, 1914, p. 103–109. The influence of NaCl

to dry, the droplets run together into larger and larger drops which are also refractile and homogeneous. Their appearance is very similar to that of a fatty substance.

These droplets persist in normal hydrochloric acid but dissolve in sodium hydroxide (0.9 per cent.). When treated with a saturated solution of Sudan III. in 96 per cent. alcohol, all of the droplets turn red and appear as bright red points. These facts indicate the lipoid nature of the droplets.

The final analysis of this substance is a task for the chemist. The important fact in connection with these experiments is that the spermatozoa are always accompanied by a variable quantity of a lipoid substance.[1] This lipoid substance is responsible for the reaction with which this paper is concerned. The lipoid is in the state of a colloidal sol. When mixed with salt solution its state of dispersion is gradually changed but it still remains in the sol state. Upon the addition of follicular fluid, the thermolabile substance in this fluid exerts an influence upon the lipoid changed by salt solution and causes it to pass into the gel state. A coagulum is thus formed. Because of these characteristics of the reaction, I have designated the latter as a lipo-gel reaction.

The question to be next considered is whether the lipoid substance which surrounds the spermatozoa is specific for the reaction as is the thermolabile substance present in the follicular fluid. An article of Kölliker's suggested to me that the lipoid accompanying the spermatozoa might be similar in nature to myelin,[2] found in the central nervous system. I therefore mixed follicular fluid with a cell-free extract obtained by grinding up spinal cord (cow) in salt solution in a mortar and filtering. The

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solution upon lipoids was observed also by Runnstrom, J., "Weitere Studien über die Verenderungen der Lipoide bei der befruchtung Seeigeleies," *Arch. Zool.*, 16, 1924, p. 1. The same effect was obtained instead of NaCl solution with thymol by Bidermann, W., "Über Wesen und Bedeutung der Protoplasma lipoide," *Pfluger's Archiv*, Bd. 202, 1924, p. 223.

[1] The same idea of a substance surrounding the spermatozoa is considered by Braus-Redenz and Redenz, H. E., Nebenhoden u. Samenfäden. Anat. Anz. Erg. 58, 1924, p. 121–131. But these authors merely postulate that such a substance exists and give no evidence concerning its nature. As regards the rôle of this hypothetical substance, the study of Redenz does not touch the essential meaning of it because the nature of the substance is not explained.

[2] A. Kolliker, "Phisiologische Studien über die Samenflüssigkeit," *Zeitschr. für wiss. Zool.*, Bd. VII., 1856, p. 201.

result was exactly the same as when follicular fluid is mixed with spermatozoa, except that the coagulum was more translucent.

Other organs which contain lipoid substances were then tested in the same way. The following were tried: thyroid, parathyroid, pancreas, adrenal gland, hypophysis, corpus luteum, skeletal muscle, heart, kidney, cerebrum, cerebellum, subcutaneous fat, subperitoneal fat, uterine mucous membrane, stomach, and lung. A positive reaction was obtained with all of these except the last three. The coagulum in all of these cases was quite similar and appeared under the same conditions as the coagulum produced by sperm and follicular fluid. Size, color, and time of formation vary a little with the organ used but the general result is the same.

Numerous experiments of this kind were performed; in each case tests were made in the slaughter-house with organs from freshly killed animals but without salt solution, and again in the laboratory in the manner described above. Sometimes when the reaction failed to occur when the tests were made at the slaughter-house, it succeeded in the laboratory after the addition of sodium chloride. Thus the reaction with different organs and follicular fluid has the same characteristics as the reaction between spermatozoa and follicular fluid.

Furthermore the typical reaction is again obtained upon mixing a commercial lipoid product kephalin (made by Armour and Company) with follicular fluid. Extracts which I have prepared from spermatozoa and medulla oblongata[1] likewise give the coagulum when mixed with follicular fluid, although the coagulum is rather thin. None of the extracts mentioned nor kephalin give any reaction with amniotic fluid, aqueous humor of the eye, or hydatic fluid.

The reaction in all of these cases invariably fails to occur if the follicular fluid is heated to 56° C.

Thus the supposition that the coagulum results from the action of a particular thermolabile substance in the follicular fluid on the lipoid material surrounding the spermatozoa is demonstrated to be correct.

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[1] Spermatozoa or ground nervous tissue were kept 24 hours in 96 alcohol, than 24 hours more in ether; evaporation on water bath. The residue dissolved in salt solution 0.9; centrifugation for elimination of cells themselves. The filtrates used for tests. *Cf.* Loewe, S., "Zur physikalische Chemie der Lipoide, I.–IV., *Biochemische Zeitsch.*, Bd. 42, 1912.

6. The question next to be considered is the rôle of sodium chloride in the reaction. It has been shown that sodium chloride accelerates the reaction. Microscopical observation of the action of sodium chloride on spermatozoa indicates that the sodium chloride causes the minute droplets of lipoid to aggregate into larger droplets. We can conclude that the sodium chloride prepares the way for the action of the follicular fluid by producing some change in the colloidal state of the lipoid. Further research would be required to elucidate this point.

7. An additional point has been determined,—namely, that the lipoid substance occurs on the surface of and between the spermatozoa. This is shown by the following experiments:

(*a*) When spermatozoa are washed once with 0.9 per cent. salt solution and filtered, both the washed spermatozoa and the filtrate produce the reaction in five to ten minutes.

(*b*) When such spermatozoa are washed a second time and filtered the washed spermatozoa and the filtrate from the second washing yield the reaction only after 24 hours.

(*c*) The same spermatozoa after being washed a third time do not give the reaction in 24 hours nor does the filtrate from the third washing yield any coagulum in this time.

(*d*) These spermatozoa after being washed three times were ground in a mortar with salt solution and filtered again. The reaction again failed.

From these experiments it appears clear that the lipoid substance is not contained inside of the spermatozoa but occurs on their surfaces and between them. The question of the rôle of this lipoid for the life of the spermatozoa and in the process of fertilization will be the subject of new researches now under way.

IV. The Physiological Significance of Lillie's Reaction.

The first point to be considered is whether the reaction is organ specific. The experiments show that only follicular fluid produce the reaction and so it is organ specific in this sense. *Stricto sensu*, the reaction is however not specific for I have shown that it can be obtained with products from a large number of organs and tissues. But as the reaction occurs in nature it may be said to be specific, for the follicular fluid under natural conditions does not meet any other fluid with which it produces a coagulum except spermatozoa. It has been shown that the reaction does not occur with peritoneal fluid, smear of the mucosa of the genital tract, or urine. The reaction is produced with lipoids but the only lipoid with which the follicular fluid would ordinarily come in contact is that accompanying the spermatozoa.

We may next discuss the possible physiological purpose of this coagulation phenomenon. At first sight the phenomenon appears to be of a paradoxical nature. In order to perform their function of fertilizing the egg the spermatozoa must retain their motility when introduced into the genital tract; yet it appears from my experiments that in the genital tract they meet a fluid, namely, the follicular fluid, which destroys their motility.[1] Without discussing this matter at too great length the following explanations of the utility of the reaction may be suggested.

1. Lillie has shown that the eggs of certain invertebrates contain and produce a substance which he designates as fertilizin which has the property of activating and agglutinating the spermatozoa and is of importance in the fertilization of the egg. If fertilizin is produced by the mammalian egg it would certainly occur in the follicular fluid. I could not determine the place of origin of the substance in the follicular fluid which gives the coagulating reaction with spermatozoa. It might originate from the ovum, or the granulosa cells. My experiments (p. 230) indi-

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[1] It is very surprising that in the whole literature one can not find the slighest indication of this phenomenon. A tremendous number of workers have tried the reaction upon spermatozoa of different kind of substances *excepting* the follicular fluid and this seems the most logical substance to be tried. Professor Lillie was the first to try this reaction and thus opened an entirely new field of experimentation. The origin of the whole question concerning the lipo-gel reaction is included in the long series of works published by Professor Frank R. Lillie. Compare, *e.g.*, "Studies in Fertilization. V. Mechanism of Fertilization in *Arbacia*," *The Journ. of Exp. Zoöl.*, Vol. 16, 1914, p. 523; "Studies on Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special References to Egg-extractives," *The Journ. of Exp. Zoöl.*, 1913, Vol. 14. As a general review of the entire problem in which my work must be integrated see Frank R. Lillie, "Problems of Fertilization," Univ. of Chicago Press. One can find there also a very rich literature concerning the subject.

cate that the granulosa cells (including presumably the ovum) do not contain sufficient of the substance to give the reaction. It was also shown that blood does not yield the reaction. It is therefore necessary to suppose that the substance responsible for the reaction is a modified product of either the blood or the granulosa cells plus the ovum, or of both, and does not exist as such in any of these objects; or that it is a substance which gradually accumulates in the follicular fluid. In favor of the hypothesis that the active substance originates from the cells of the follicle including the egg is the observation that follicular fluid from atretic follicles does not yield the reaction. The ovary of the cow frequently contains follicles filled with a clear colorless liquid; such follicles do not contain any granulosa cells or any ovum. They are readily recognized by the colorless watery appearance of the contained fluid. This fluid however invariably fails to yield the coagulating reaction, eleven cases having been tested.

The active substance in the follicular fluid does not appear to be identical with the fertilizin of Lillie in its properties. For instance, fertilizin is stated to be very resistant to heat, while the follicular substance as shown above loses its coagulating property when heated to 56° C.

2. Whatever may be the rôle played by the coagulating substance in the process of fertilization I believe that it may have another secondary function which does not necessarily exclude the first suggestion. I am inclined to think that the follicular fluid may serve to protect the peritoneum against possible infection by infected spermatozoa.

I have made a number of observations on the life and behavior of spermatozoa in various media and particularly in follicular fluid. From these experiments it appears that the spermatozoa furnish a very good medium for the growth of bacteria. After standing for three or four hours at room temperature sperm suspensions were seen to contain many cocci and bacilli: and after twenty-four hours they were swarming with microörganisms. For this reason spermatozoa live much longer at low than at higher temperatures. Spermatozoa will live for several days in small glass tubes if they are kept in the refrigerator, but only for

24 to 30 hours at room temperature. On two occasions spermatozoa that had been kept in the refrigerator for nine days showed motility when brought into room temperature.[1] Spermatozoa removed and kept under aseptic conditions may remain alive for eight days in the refrigerator and for four or five days at room temperature. Even infected spermatozoa retain their motility for some time and move through the field of the microscope carrying bacteria with them. I have injected spermatozoa from the ram into the excised uterus of a sheep. In two hours the spermatozoa were found to have penetrated into the uterine tube but they were greatly damaged and fragmented and many of them had been phagocitized. Those spermatozoa which were still alive were in a state of agitation and covered with bacteria. It appears that the surface of the spermatozoa is very sticky and that all kinds of particles therefore adhere readily to them.[2]

The spermatozoa are placed normally in a very infected organ, namely, the vagina. The penis itself bears some species of bacteria and at all events at the moment of copulation many bacteria are introduced from the outside into the vagina. These bacteria are carried up into the uterine tubes by the spermatozoa and constitute a menace for the peritoneum which is well known to be exceedingly sensitive to infection. The agglutination of the spermatozoa by the follicular fluid would hold these bacteria and permit them to be phagocytized more readily, thus protecting the peritoneum from possible infection.

This hypothesis of the protective rôle of the coagulating substance in the follicular fluid does not exclude the possible importance of this fluid in fertilization; also it is not possible, at the present time, to estimate more accurately the practical efficacy of this protective rôle of the coagulating substance.[3]

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[1] The same observation on favorable effect of low temperature upon longevity of spermatozoa has been repeatedly made *e.g.* by Redenz in the paper already mentioned; *Cf.* also Mettenheimer, M., "Sperma und kunstliche Befruchtung bei Mensch und Tier," *Münch. Med. Woch.*, schr. 72, Jahr. g. 1925, p. 977; Mettenheimer, M., *Arch. f. Gynak.*, 1925, Bd. 162, p. 215.

[2] I found after I finished my work, that the proof that spermatozoa may transport bacteria was already furnished by Rotter, "Wie ascendiert die Gonorrhöe," *Archiv f. Gynäkologie*, Bd. 117, S. 153. This author has proved that spermatozoa can transport *Bacillus subtilis* and *gonococcus.*

[3] I am obliged for editing this paper to Dr. Libbie Hyman to whom I express my best thanks.

THE DISTRIBUTION OF SUBSTANCES IN THE SPERMATOZOÖN (*ARBACIA* AND *NEREIS*).

STUDIED BY INTRA VITAM STAINS AND BY STAINS OF LIPOIDS ACCORDING TO THE METHOD OF SCHUMACHER.

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I. INTRODUCTION.

In a previous paper [2] I come to the conclusion that the spermatozoa of mammals are surrounded by a layer of a lipoid substance which is responsible for a reaction observed by Prof. Frank R. Lillie, and called by me the *lipo-gel reaction*.

With other methods and in different animal species, I have been able to analyze much more completely the distribution of this lipoid substance in and between spermatozoa. The following description is a record of these new facts.

In a recent paper Josef Schumacher has devised methods for the identification of lipoids, lipoproteids and fats in the tissues and cells by means of combining different strong reagents with the action of stains. One conclusion of his work is that there is a series of stains (above all Victoria blue and fuchsin) which have a special affinity for lipoids, giving a salt by combination with the lipoidic acid. After destroying the proteins by different macerating reagents, one can isolate the lipoids and in this case the staining with the mentioned substances is a specific stain.

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[1] From the *Marine Biological Laboratory, Woods Hole, Mass.*

[2] Gregor T. Popa, 1927, "A Lipo-gel Reaction Exerted by Follicular Fluid upon Spermatozoa (Lillie's Reaction) and Its Significance, BIOL. BULL., Vol. 52, p. 223.

I shall not review this method in detail. Everyone who needs to use it must read the original paper: Josef Schumacher: "Zur Chemie der Zellfaerbung VIII. Mitteilung. Ueber die Nachweis der Lipoide in Zelle und Gewebe," Chemie der Zelle und Gewebe, *Zeitschr. f. die Probleme der Gaerhung, Atmung u. Vitaminforschung*, Bd. XII., Heft 5, 1926, S. 433.

I have used his methods (see page 247) but most of the observations were made by simpler methods, using stains recommended by him as specific stains, and in addition various intra vitam staining methods. In the latter case the staining was done under the cover slip as in the method of Koltzoff "Studien ueber die Gestalt der Zelle," *Arch. f. Zellforschung*, Bd. II., S. 1, 1909.

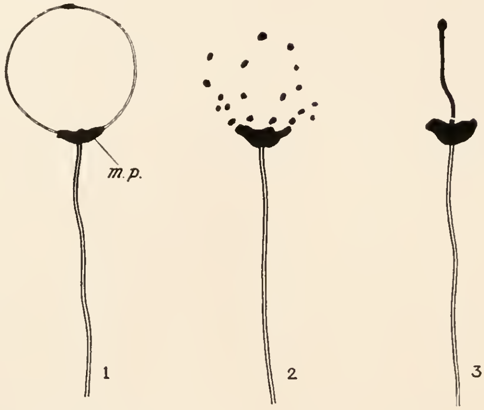
II. RESULTS.

1. The Use of Stains with Fresh Spermatozoa.

(*a*) *Victoria Blue and Fuchsin.*

Victoria blue in 1 per cent. solution in distilled water: Stain under the cover slip: one drop of sperm suspension (one drop dry sperm to 10 cc. sea-water) is placed on the slide, and after covering with a cover slip, with a fine pipette one adds at the margin of the cover slip a drop of staining solution. The fluid penetrates by capillarity, advancing more and more slowly. In this way, one gets in the same preparation all degrees of staining, from the strongest color to the complete lack of stain. At the one end of the slide the spermatozoa are overstained, and on the opposite side the spermatozoa are still active.

The first impressive fact is a remarkable change of the shape and size of the spermatozoa. The stain is in distilled water and the spermatozoa are in sea-water: there is a great difference in the osmotic pressure of the two fluids. Thus, a rapid penetration of the staining solution takes place into the spermatozoa, which stain immediately. Shortly after, the heads of the spermatozoa swell, become round and then burst; the following diagrammatic figures show some of the variations in distribution of the stain (Figs. 1–3).



FIGS. 1, 2, 3. Fresh spermatozoa of *Arbacia* stained with 1 per cent. solution of Victoria blue in distilled water.

When the reaction is too strong, *i.e.*, when the contact of the spermatozoa (in sea-water) with Victoria blue (in distilled water) is immediate, the tails swell also from place to place and one may find shapes as in Figure 4. Frequently, when the reaction is still stronger, the tails twist round the heads and shapes very similar to those described by Koltzoff result (*loc. cit.*, pp. 9–12).

Victoria blue, fuchsin, and the dyes from the same series (Gentian violet, Malachite green, etc.) always stain substances in the acrosome region, in the middle piece, and in the tail. The reaction with Victoria blue, and with the stains in the same class, gives strong reasons for postulating the presence of lipoids in the composition of the acrosome, middle piece, and tail.

Victoria Blue in Alcohol (1 cc. of the distilled water solution in 10 cc. alcohol, 70 per cent.): The solution fixes the spermatozoa at the same time that it stains, and the stain being more dilute, one can see the gradual transformations of the spermatozoa. The heads swell slowly; the tails never swell. The distribution of the stain in the spermatozoa is exactly the same; tip, middle piece, tail. Also, the stain in the region of the acrosome does not always occur, and the middle piece varies in size. By variations of concentration one can have a series of pictures more and more similar to those obtained by Victoria blue in distilled water.

Fuchsin (1 per cent. distilled water solution): The affinity of the fuchsin for the lipoids is less than that of Victoria blue. The staining is accordingly slower and one can follow this process more gradually in the parts of the slide where the effect of distilled water is moderated by a suitable mixing with sea-water.

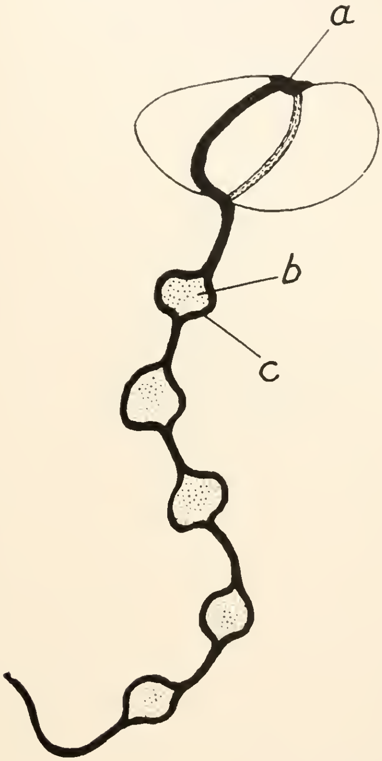


FIG. 4. *Arbacia.* Fresh spermatozoön in 1 per cent. Victoria blue in distilled water—Swelling of tail and twisting around head. *a*, middle piece; *b*, hydrophilic substance; *c*, lipophilic substance.

The new fact obtained by this method is the possibility of observing *changes of shape and variations of size and form of the middle piece.* One can see in its region only granules varying in number (2–6), or a ring varying in size from one spermatozoön to another, and in the same spermatozoön during the observation[1] until at the end of the reaction almost all spermatozoa show the middle piece as a compact mass, red-stained.

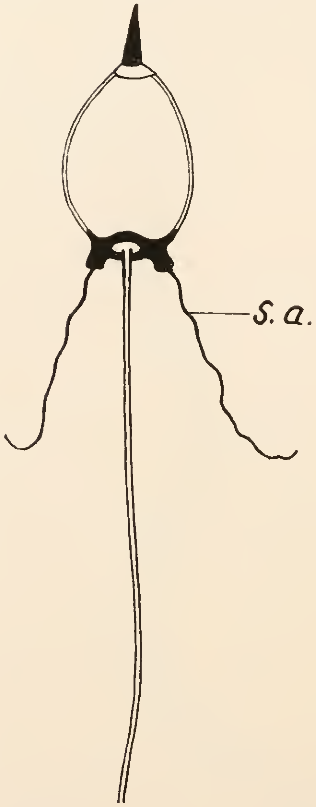


FIG. 5. Spermatozoön of *Nereis* to show sensillæ amœboideæ.

When the slowly moving spermatozoön shows its tip, one can see that there is really a minute opening. The tip of the spermatozoön is extremely sticky and adheres to everything which touches it: granules, slide, cover slip, eggs, or to another spermatozoön. Frequently it sticks to the tail of the same spermatozoön.

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[1] The instability of this region was very clearly observed by Retzius and Ballowitz, but they believe that it occurs because of the technique. However, without staining, under conditions approximately normal, the same variations of shape can be noticed. Gustav Retzius, "Die Spermien von *Aurelia aurita* L.," *Biol. Unters.*, N. F., XIV., 1909, S. 67. Gustav Retzius, "Die Spermien der Nereiden," *Biol. Unters.*, N. F., XIV., 1909, S. 69. E. Ballowitz, "Über die Körnige Zusammensetzung des Verbindungsstückes der Samenkörper der Knochenfische," *Arch. f. Zellforschung*, 14 Bd., 1917, p. 355.

Victoria blue or *Fuchsin* in sea-water solution: These substances are very feebly soluble in sea-water; but if we keep an excess of stain for several hours in sea-water, a weak solution is obtained in which the spermatozoa can live for ten to thirty minutes and one can see the changes which occur more easily than in distilled water solution.

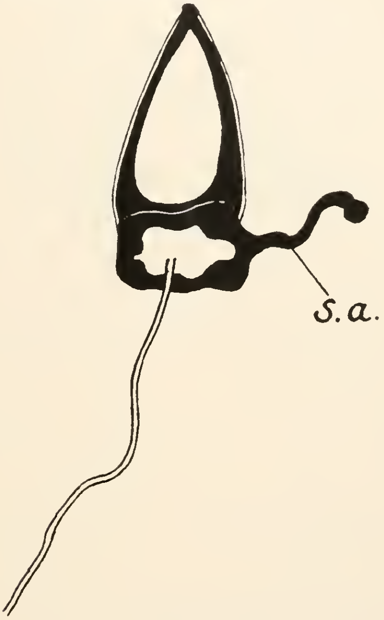

FIG. 6. Spermatozoön of *Arbacia* showing sensilla amœboidea.

The regions of the spermatozoön already mentioned take the color. But for a short time there is a slight stain on the surface of the head also, before the middle piece is stained. After a variable time the head loses the color gradually and one can see a slow enlargement of the middle piece and the formation of delicate protoplasmic filaments therefrom, which move sporadically. These deserve a more precise description.

The basal part of the head has the appearance of a ring, somewhat thicker than the rest of the head. When the spermatozoön turns in the fluid in such a manner as to show its larger end, one can see very clearly even on fresh preparation, not stained, the presence of this ring. It has different sizes, according to the species (*Arbacia* and *Nereis*) and different outlines on the same spermatozoön. Here and there it is thicker or thinner; sometimes it becomes fragmented in granules and again, it forms a compact mass. In *Nereis* (Fig. 5) there are normally two swellings placed symmetrically, outgrowths of the ring, on which one can distinguish two long filaments as in Fig. 5. In *Arbacia* (Fig. 6), the ring shows in the same way various thickenings (3–5) and these, not so constantly as in *Nereis*, support (usually only one of them) a prolongation ended by a kind of knob.

As the spermatozoön floats in the fluid, these filaments show protean movements, elongation, shortening and thickening, very similar to amœboid movements. They are constructed of the same material as the basal ring and they are to be considered as prolongations of this. Provisionally they may be called *sensillæ amœboideæ*.[1] In all changes of osmotic pressure, in all media which change or kill the spermatozoa, these sensillæ retract upon the ring where they produce granules projecting a little above the general level.

(*b*) *Janus Green; Trypan Blue; Neutral Red*
(*1 Per Cent. in Sea-water*).

These substances are frequently used for staining intra vitam. The penetrating power is different for each. Janus green gave the best results in our experiments. It penetrates very slowly, and shows excellent contrasts.

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[1] It is very interesting that the presence of these filaments was noticed by Mischer "Die Spermatozoen einiger Wirbeltiere. Ein Beitrag zur Histochemie," *Verh. der naturf. Gesellsch. in Basel*, 1878, Bd. VI.; the interpretation of them was wrong. E. Ballowitz, in the same species, denies Mischer's observations "Über die Samenkörper des Lachses. Ein weiteres Beitrag zur Kenntnis der Spermien der Salmoniden," *Arch. f. Zellforschung*, 14 Bd., 1917, S. 451; but in an older paper and in other species, "Untersuchungen über die Struktur der Spermatozoen, zugleich ein Beitrag zur Lehre von feineren Bau der Kontraktilen Elemente. Die Spermatozoen der Insekten," *Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317, he himself has observed such filaments and even their movements. He even calls them *Wimpelfasern*. The meaning of them is not explained, and he says that they are "*rätselhaft*." The conditions of observation were bad because he employed always "maceration," after killing the spermatozoa. In fact, the sensillæ are to be observed much more clearly on fresh, slowly motile spermatozoa, without stain.

By these stains one obtains more clearly the same results as by Victoria blue in sea-water. With Janus green especially, one obtains an entire series of pictures, which prove clearly that *there is a migration of the same substance which stains in Victoria blue*, to the connecting region between head and tail and a large accumulation there of the migrating substance (Fig. 7 *a–e*). But here and there one can find spermatozoa in which the lipochromatic substance[1] migrates towards the apex. Compare the action of Victoria blue and fuchsin in distilled water (Figs. 2, 3, and 4) in which one sees the lipochromatic substance also accumulated on various regions of the head. These facts show the presence in the spermatozoön of *an active cytoplasmic layer*, external to the nucleus, *loaded with lipoids* and *taking*

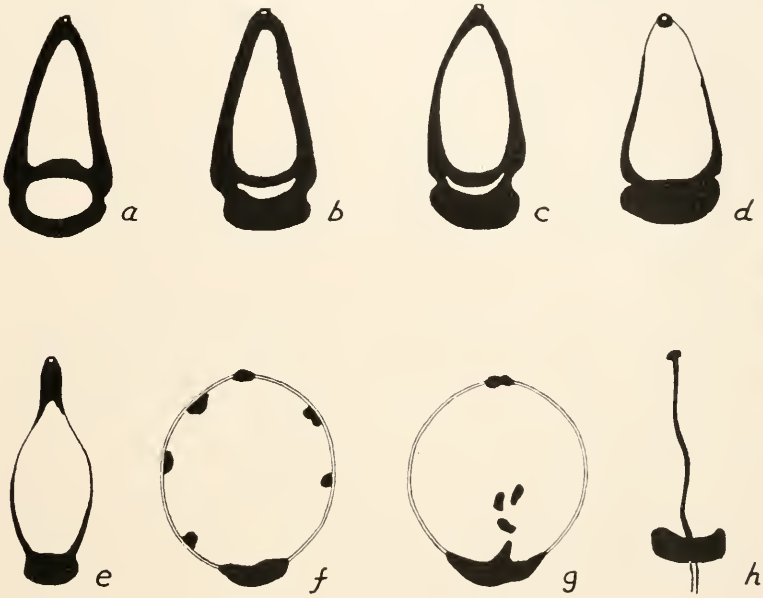

FIG. 7. *a–e*, sperm heads of *Arbacia* showing stages of staining in Janus green in sea-water; movements of lipochromatic substance. *f–h*, Victoria blue in sea-water, various conditions. The lipochromatic substance is shown black.

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[1] I use this term *lipochromatic substance* for a succinct description, implying only that the substance mentioned takes very strongly the stains reputed by the work of *Schumacher* to be lipophiles: Victoria blue and fuchsin especially.

various forms. This layer flows in and out of middle piece and forms the sensillæ.[1]

2. Staining Methods after Fixation.

Spermatozoa of *Arbacia* and *Nereis* were also studied by the more usual methods of fixing and staining. The fixing methods used were:

1. Corrosive sublimate, saturated solution in distilled water, 100 parts, plus glacial acetic acid, 8 parts.
2. Vapor of osmic acid, 2 per cent. aqueous solution.
3. Flemming, strong solution.
4. Formalin, 10 per cent.
5. Heat, after rapid evaporation.

The following stains were employed:

1. Iron-hæmatoxylin (Heidenhain) and eosin.
2. Iron-hæmatoxylin (Heidenhain) and safranin.
3. Fuchsin.

These procedures applied to the study of the spermatozoa, when united with the observations on living material, give us the conviction that *there is no one fixing fluid which preserves perfectly the form and the structure of the spermatozoa.*[2] The fastest acting fixing agents (as vapor of osmic acid) and the most penetrating fluids (as acetic acid plus *k*-bichromate) change the size, the configuration of the spermatozoa and the mutual relations of the substances in it. The outlines of the head slightly change, the heads swell, the sensillæ disappear, the ring changes its shape, and the lipochromatic substance migrates.

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[1] I have strong reasons to believe that Ballowitz has observed also the migration of the peripheral substance of the spermatozoa, "Untersuch. über die Struktur der Spermatozoön der Fische. Amphibien, Reptilien," *Arch. f. mikr. Anat.*, 36 Bd., 1890, S. 225. His explanation is not satisfactory. He believes that the change of color in various parts of the head of the spermatozoön is a consequence of stain diffusion in the medium (p. 239). But the changes can be better observed by the method of Victoria blue, fuchsin and Janus green than by his method; and one can also observe the accumulation of the substance in the connecting region even on spermatozoa not stained at all.

[2] Students of spermatozoa note the same thing. This fact is demonstrated particularly clearly by C. Pictet, "Recherches sur la spermatogénèse chez quelques inverté brés de la Méditerranée," *Mitteil a. d. Zoöl. St. Neapel.*, 10 Bd., 1891–93, S. 75.

3. Schumacher's Methods.

A.

One makes a smear with sperm as thick as possible, and before the sperm is dried the slide is plunged into HCl solution 1 : 4. The slides remain in the acid 24 hours. After that time, wash in distilled water and stain for 5 minutes in 1 per cent. Victoria blue in distilled water. Again 5 minutes in distilled water. Mount after usual treatment in cedar oil, or, in order to have clearer pictures (but more unstable) one can mount directly in water and seal with paraffin.

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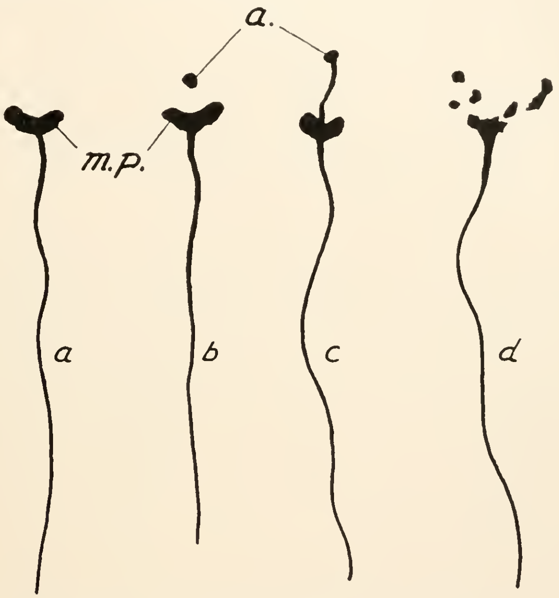

Fig. 8. Spermatozoa of *Arbacia* prepared according to method A of Schumacher and stained in Victoria blue.

The idea of this treatment is as follows: The various chemical components of cells show a different resistance to the various chemical agents. By combining the destructive agents and the time of acting, it is possible to isolate by turn the chemical components of the cell and detect them afterwards.

Schumacher uses strong acids in order to destroy the proteins of the cell. The remainder, after maceration, must be some substance not influenced by acids, essentially lipoproteins (proteins united with lipoids) and lipoids. If this is true, the stains characteristic for proteins, such as methylen blue, will stain no more, and on the contrary, the stains of the fuchsin series still will give a coloration.

This method was used by Schumacher in studying leucocytes, yeast cells, liver, lung, and skin. I have applied this method in studying the spermatozoa.

With the above method one obtains with Victoria blue the following pictures (Fig. 8); staining with methylene blue fails completely. This demonstrates that the various portions of spermatozoa stained with Victoria blue by the procedures already described, resist the action of strong acids, while the other portions are destroyed. Therefore, according to the theory of Schumacher, we can say that *in the construction of the tail, middle piece, and acrosome of the spermatozoön there is a great quantity of lipoid substance.*

B.

For deciding if in an organic construction, which resists the action of acids and still is stained by Victoria blue, there are free lipoids or lipoids associated with proteins (lipoproteins), Schumacher uses another procedure: Smears made as in *A* (above) are placed for 24–36 hours into a mixture of alcohol and ether. Afterwards the slides are transferred for 24 hours to HCl 1 : 4. The staining following is identical with that described in *A* (above).

The idea is this: The alcohol-ether dissolves and washes out *free* lipoids, but does not touch the lipoproteids; afterwards HCl destroys the proteins, and again the lipoproteids remain intact, and thus they are isolated. If, after such a treatment, we still obtain a coloration, we can say that there is a lipoprotein component.

Applied to the study of spermatozoa, this method gives the pictures shown in Fig. 9. A pale reticulum built by interlacing tails, and here and there some knobs at the ends of fibers, inferred to be the remains of middle pieces. It is to be noticed also that the fresh preparations immediately after staining with Victoria blue, are much clearer than the preparations mounted in balsam, and particularly after treating with xylol.

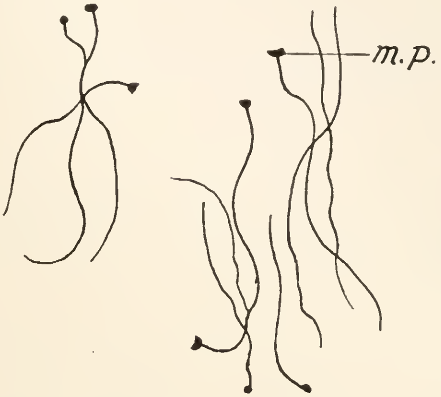

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FIG. 9. Spermatozoa of *Arbacia* prepared according to method B of Schumacher and stained in Victoria blue.

Summarizing the facts obtained by the method of Schumacher, one can say that in the structure of the spermatozoön there are free lipoids and lipoproteids which are located normally in the tail, middle piece, and acrosome. The facts described in 1 and 3, connected with the facts observed in living spermatozoa, demonstrate that the lipoids and lipoproteids of the tail are located *on the surface.*

4. OSMIC ACID (1 PER CENT. AQUEOUS SOLUTION 24 HOURS, OR 2 PER CENT. AQUEOUS SOLUTION FIXING BY VAPOR 1–5 MINUTES).

Osmic acid shows in *Arbacia* very similar pictures to those obtained by the lipophilic stains. One can see very clearly the basal ring, the tail, the middle piece; the conical part of the head appears darkened and unburnished, which gives us the impression that the substance darkened by osmic acid is located on the surface of the spermatozoön. The substance between spermatozoa is darkened too. On fresh preparations this substance can be distinguished still more clearly. If spermatozoa are shaken in sea-water, and from the clear fluid, which separates in the upper part of the test tube, a drop is taken and submitted to the action of osmic acid vapor, one sees under the microscope very fine round droplets, black and refractile, evidently originating in the intercellular substance. The preparations by osmic acid give us the impression that there is *on the surface of and between the spermatozoa a substance which contains fat* and which surrounds the cells as a mantle.

5. Sudan III.

Sudan III. shows in the same way as osmic acid, the presence of fatty globules accumulated between the spermatozoa after treating them with the reagents.

6. The Macrochemical Test with Victoria Blue.

If 1 per cent. Victoria blue in distilled water is mixed with ether, one obtains, after shaking, an emulsion which, after 10–30 minutes, separates in two distinct layers: the *non*-stained ether above, and the strong colored Victoria blue below. If to the same mixture one adds dry sperm, after shaking, the ether separates again, but now it carries with it the fatty substances from the spermatozoa. These substances are already stained by Victoria blue, and, therefore, the ether in the upper part of the test tube is blue. If, instead of spermatozoa, we use only the fluid separated after centrifuging a sperm suspension, we may have a complete transfer of color into the ether, the lower layer, representing the water of the Victoria blue solution, being now uncolored. This reaction corroborated by the tests of Schumacher and the reactions with Sudan III. and osmic acid demonstrates that around and between spermatozoa there is a substance rich in unsaturated and neutral fats.

7. Observations on Living Spermatozoa in Sea-water.

Observations are made with the oil immersion lens. By varying the focus, the strength of the light and the eye pieces, one can see quite clearly many of the facts obtained by staining methods. Without doubt, the value of this kind of observation is very great, especially when combined with the above mentioned methods; more and more one reaches the conviction that there are in the spermatozoa two different zones: a darker one at the periphery, which becomes thicker towards the connecting region between head and tail, where it constructs a large ring. The central zone is more refractile. It is larger towards the connecting region and diminishes towards the apex.[1] Almost all transformations of the ring can be seen in fresh spermatozoa; the sensillæ amœboideæ show most clearly. The migration of substances can be deduced by seeing the changeable sizes of various parts of the *same spermatozoön* during the observation.

In the preceding observations, there is one fact which deserves more emphasis: this is the *stickiness of the pointed part of the head, and of the connecting region too.* Very frequently, and chiefly in some particular reactions, taken into consideration below, the spermatozoa bunch together, sticking by these two regions. By careful and patient observations of fresh spermatozoa, during longer time and in various media, one gets the impression that the spermatozoa eliminate through the point of the head (where really there is an exceedingly minute opening) very small amounts of an extremely sticky substance.

8. The Effects of Egg-water on the Spermatozoa.

The mutual relations of the various constituents of the spermatozoön, being established and knowing the lability of these relations, a large field for studying the topographical changes of the principal substances in various media is opened. I shall limit myself here to one point: *the changes which occur in the spermatozoa in egg-water, which might be expected to resemble changes preliminary to fertilization.*

This is the classical phenomenon studied by Lillie.[2] It is very well known at the present time that there is exerted an agglutinative action upon spermatozoa by the sea-water in which sea-urchin eggs have been for a short time. The reaction is instantaneous. The spermatozoa exhibit great activity and bunch together, for a while, in compact clusters of various sizes.

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[1] Mischer (*loc. cit.*) noticed the same thing. Ballowitz in *Untersuch. u. die Struktur d. Sp.* (Fische, Amph. Rept.), p. 249, distinguishes in the head of the spermatozoön two different zones, and he says that in the process of swelling "the internal zone leads." In fact, it alone swells the external substance merely changing its place.

[2] Frank R. Lillie, "Studies of Fertilization. VI. The Mechanism of Fertilization in *Arbacia*," 1914, *Journ. of Exp. Zoöl.*, Vol. 16, p. 523. Frank R. Lillie, "Studies of Fertilization. V. The Behavior of the Spermatozoa of *Nereis* and *Arbacia* with Special Reference to Egg-extractives," *Journ. of Exp. Zoöl.*, Vol. 14, 1913, p. 515.

What is the immediate cause of the agglutination residing in the spermatozoa? Is it due to a change in the arrangement of the substances, or to an active elimination of the sticky substance observed in fresh suspensions of spermatozoa?

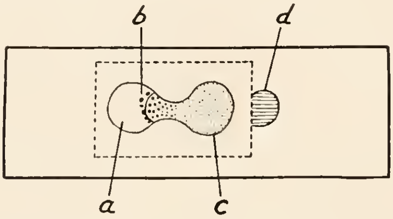

FIG. 10. Arrangement of agglutination experiment; *a*, egg-water. *b*, clusters. *c*, sperm-suspension. *d*, drop of Janus green.

One drop of egg-water and one drop of sperm suspension are placed on a slide (Fig. 10). A very narrow bridge is established between the two drops. Immediately the agglutination takes place where the drops meet, and slowly spreads, so that we have again various stages of a phenomenon on the same slide.

At the left end of the egg-water drop (Fig. 10) the fluid is still clear; at the middle, agglutinated clusters of spermatozoa in great activity; at the right side of the sperm suspension drop, the spermatozoa in various degrees of motion, are in a homogeneous suspension.

In these conditions, one keeps the slides various lengths of time (1–10 minutes). Afterwards one puts very carefully a cover slip with another drop of egg-water underneath (to mitigate the power of capillarity). Many bunches of spermatozoa are spread out, but many other resist, and one can observe, under the oil immersion lens, what happens in the clusters.

A long series of transformations of the individual spermatozoa occur, represented by the next sketch (Fig. 11). These transformations take place under the influence of egg-water only. But in order to see them more clearly, one may add at the margin of the cover slip a drop of 1 per cent. Janus green in sea-water. The changes shown diagrammatically in Fig. 11 are: (*a*) A normal spermatozoön; (*b*) Initial changes in the arrangement of lipochromatic substance; (*c*) The nucleus swells a little and moves towards the base of the head. At the same time the lipochromatic substance divides into two, a part follows the nucleus, covering it, another part remains in the region of the basal ring. Thus one can see in fresh preparations *on the connecting region* of the spermatozoa two transversal dark zones, between which there is a refractile one. On stained preparations

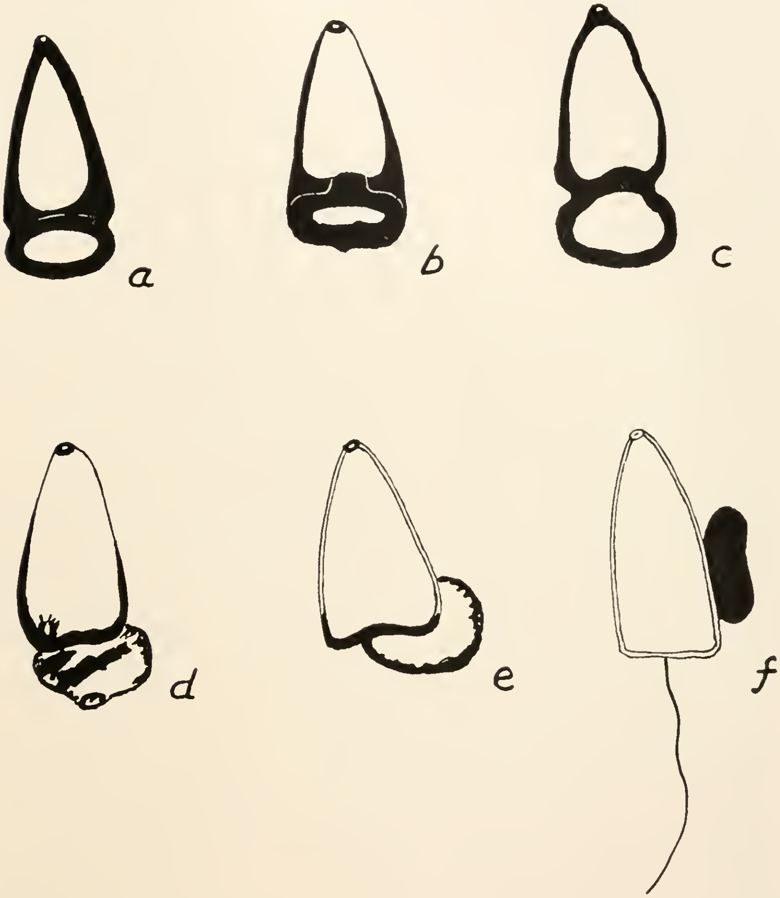

FIG. 11. Behavior of the spermatozoa of *Arbacia* in egg-water; intra-vitam staining with Janus green. See text.

there are two colored strips and one light band; (*d*) The same process, much more advanced; (*e*) The lipochromatic substance accumulated in greater amount, shifts from the connecting region; (*f*) A round or slightly oval body is thus extruded from the head, and it takes a lateral position, where it persists without any modification, and the whole spermatozoön becomes perfectly quiet. It is to be noticed that the spermatozoa which have extruded this *lateral body* do not swell. On the other hand, the spermatozoa which have not extruded it, swell and become round.

III. DISCUSSION.

Summarizing all the facts revealed by the various methods above described, concerning the topographical distribution of the material in the spermatozoön (in *Nereis* and *Arbacia*) there is to be distinguished a substance stained by lipophilic stains, *the lipochromatic substance;* and another substance very avid for water, *the hydrophilic substance.* The lipochromatic substance is located at the periphery of the spermatozoön, and its thickness is variable in different regions; towards the connecting region it constructs a large ring and the sensillæ; in the tail it takes part in the construction of the tail mantle. This substance is not avid for water, and therefore does not change its volume in various hypotonic solutions. On the other hand, it shifts position readily, and it accumulates normally in the connecting region of the spermatozoön (Fig. 11).

The hydrophilic substance is located in the axis of the spermatozoön, and does not take the lipophilic stains. In the head it includes the nucleus or may be represented exclusively by the nucleus. It is extremely hydrophile, swells in water (hypotonic solutions) and thus enlarges the head of spermatozoön until more than three times diameter. This substance is centrally located in the head and also in the axis of the tail.[1]

Apparently the hydrophilic substance of the head is nothing else than the nucleus and the lipochromatic substance must be the cytoplasmic component of the spermatozoön loaded with lipoids and lipoproteids.

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[1] Concerning the sensitiveness of these cells to the changes in the osmotic pressure see: Richard Goldschmidt, "Kleine Beobachtungen und Ideen zur Zellenlehre III Die Bedeutung der atypischen Spermatozoen," *Arch. j. Zellforsch.*, 14 Bd., 1921, S. 290. Richard Goldschmidt, "Versuche zur Spermatogenese in vitro," *Arch. f. Zellforsch.*, 14 Bd., 1921, S. 421. Josef Speck, "Neue Beitrage zum Problem der Plasmastrukturen," *Zeitschr. f. Zellen- u. Gewebelehre*, 1 Bd., 1924, S. 278.

The cell is surrounded by a very thin membrane, the presence of which is obvious when the heads swell and explode in hypotonic solutions. The membrane must be very elastic and permeable to water. The whole complex is protected externally by a thick *layer of fatty substance*, darkened by osmic acid and detected also by Sudan III. and by macrochemical analysis with Victoria blue.[1] Its chemical composition is still not clearly demonstrated.

Mixing dry sperm with sea-water one can clearly see this substance spreading out on the surface film like oil. In the dry sperm under the microscope, one can see how abundant this intercellular substance is. In this medium the spermatozoa live longer than without it. Presumably the contact of the spermatozoa with the water is very much mitigated by the surrounding fatty medium, so that changes of osmotic pressure influence the spermatozoa gradually, and the modifications of form occur very slowly. This layer of fatty substance may be considered on the whole as a *buffer* between a very sensitive structure (spermatozoön) and a salt solution at a very high concentration (sea-water). Certainly the fatty substance may have some other important significance, too, not yet taken into consideration.

As soon as the concentration of the medium is changed and the fatty medium is modified, the spermatozoa react first by motility, and secondly the mutual relations of the two substances (hydrophilic substance → lipochromatic substance) undergo changes. As a result we have the various pictures of the spermatozoön already demonstrated.

For completion of the general sketch of the spermatozoön there is to be remarked the presence at the distal end (that is to say, the tip of the head) of a minute opening, which it is convenient to call by analogy *micropyle;* and also at the connecting region there is another small ring very easily colored by stains which have affinity for chromatin, especially by *carbol-pyronine.* This is the classical proximal centriole.[1]

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[1] This layer outside the spermatozoön has been frequently noticed recently by various authors: Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodens," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 103 Bd., S. 391. Gellhorn, E., "Physiologische Untersuchungen an Spermatozoen und Eiern. Ein Beitrag zum Befruchtungsproblem. Sammelreferat," *Arch. f. Mikr. Anat. u. Entw. Mech.*, 1924, 101 Bd., S. 437. Redenz, E., "Versuch einer biologischen Morphologie des Nebenhodes. II. Die Bedeuting elektrolytarmer Losungen für die Bewegung der Spermien," Wilh. Roux' *Arch. f. Entw. mech. d. Org.*, 106 Bd., 1925, S. 290. T. von Lanz, "Über Bau und Funktion des Nebenhodens und seine Abhängigkeit von der Keimdruse," *Zeitschr. f. die ges. Anat. Z. f. Anat. u. Entw. g.*, 80 Bd., Festschr. f. S. Mollier, 1926, S. 177.

We have seen above the various changes of the mutual relations of the two substances in the head of spermatozoön. The end result of these changes is the elimination of the *lateral body.* This elimination phenomenon occurs, also, under various other circumstances, for instance, when spermatozoa are in weak formaldehyde solution, or in some staining solutions, but the process takes place very slowly and quite irregularly. On the other hand, in the egg-water after five minutes almost all spermatozoa show this *lateral body.*

Fresh preparations of spermatozoa of *Arbacia* in egg-water also show quite clearly the elimination of a substance through the pointed apex of the spermatozoön. This appears as a small granule on the points of almost all spermatozoa. The adhesion of the spermatozoa to one another or to other objects is made by means of this granule.[2] In the middle of some clusters one can see groups of such granules very refractile, round and close together. For this observation only fresh preparations are to be trusted, because stains may form very fine precipitates which cannot be distinguished from drops of the eliminated substance.

Very probably, under the influence of some chemical substances included in the egg-water (Lillie's fertilizin?) spermatozoa eliminate through the micropyle a sticky substance, which so long as it still adheres to the apex of spermatozoa keeps them agglutinated. After it is lost from the tip, the spermatozoa spread out again in the fluid. I cannot decide if this substance exists as such in the spermatozoön or if it is produced by a secretory process at the moment of stimulation by egg-water, as in the sense of Bowen.[3]

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[1] Here also is the opening observed by Mischer and Ballowitz (*loc. cit.*) and called by the former worker microporus.

[2] Ballowitz, E. (*Zeitschr. f. wiss. Zoöl.*, 1890, Bd. 50, S. 317) called this granule "Spitzenknopf" and noticed its instability (p. 375).

[3] According to R. H. Bowen, who adopts the theory of Nassonov concerning the rôle of Golgi apparatus, "Das Golgische Binnennetz und seine Beziehungen zu der Sekretion. Untersuchungen über einige Amphibiendrusen," *Arch. f. mikr. Anat.*, 97 Bd., 136, the acrosome should be a secretory organ and the granule on

The spermatozoa also undergo in the egg-water more extensive alterations, which are expressed by changes in the arrangement of the substances and as the end result we have the formation of the *lateral body* as described above.

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NESTING HABITS OF SOME ANTHIDIINE BEES.

CLARENCE P. CUSTER AND CHARLES H. HICKS.

The habits of the Anthidiine bees have been studied by Fabre (2), Friese (3), Davidson (1), Melander (7), Newberry (8), Hungerford and Williams (6), Hicks (5), and others. Fabre early considered the genus *Anthidium* to consist of two groups, divided primarily on habits. One he termed "cotton-workers"; the other "resin-workers." The former now comprise the genus *Anthidium;* the latter *Dianthidium.* The habits of these bees have not been studied so thoroughly in America as in Europe. Studies of *Anthidium porteræ* Ckll. have been made by Hungerford and Williams (6) and Hicks (5), and *Dianthidium sayi* Ckll. by Hicks (5).

The writers are indebted to Dr. T. D. A. Cockerell and to Dr. Francis Ramaley for helpful suggestions and for the determination of specimens; to Dr. Edna Johnson for the determination of some plants; and to Mr. S. A. Rohwer for determining some wasps.

We continued a study of these bees during the summer of 1926. The following is an account of our work.

Anthidium porteræ Cockerell syn. *maculifrons* of authors, not Smith.

Hungerford and Williams (6) state that two females of this bee were observed nesting in a bank of coarse, quartz sand near a colony of bembecid wasps. The down of the cells of the nest was thought to be derived from thistle (*Cirsium*) stems or from the inflorescence. The tunnel of one was 10 inches long.

Most of our study of this species has been done at Point East[1] and at Base Line Lake,[2] near Boulder, Colorado. The nests have been found both on hillsides and on level ground, usually in bare areas.

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[1] A small hill rising above the plains, 3 miles east of Boulder.

[2] Six miles east of Boulder on the plains.

We attempted to observe whether or not *A. porteræ* digs her own nest or uses one already constructed by another insect. Individual females have been observed as long as 15 minutes at a time entering, spending a few seconds within, and then leaving various types of holes. At other times the bee merely looks in and leaves. We have never seen this or any other species of *Anthidium* digging in the ground.

Sharp (9) states that the species of *Anthidium* do not form burrows for themselves, but use suitable cavities formed by other insects in wood, deserted nests of other bees or empty snail shells.

As far as can be ascertained, no one has before observed an *Anthidium* bee to drive away another insect and take the nest, although it has been generally assumed that it uses the deserted nests of another insect, or a convenient cavity.

While observing a wasp, *Odynerus dorsalis* Fabr.,[1] building and provisioning a nest, *A. porteræ* was seen to drive away this wasp and appropriate the nest for her own. A few days previous to August 2, a female of this wasp[2] had been observed working and nesting. During the morning of August 2, I had been watching this wasp excavating and carrying out moist pellets of earth. Shortly after noon she had apparently finished the nest. The following notes were taken in the field near Base Line Lake between August 2 and 5, 1926 (h).[3]

2 : 50′. See *Odynerus* returning, flying and carrying large lepidopterous larva. She enters hole with it, staying but a minute within, then leaves.

3 : 17′ 05″. Back without larva. Flies in wide circles after which alights near hole, tapping edge with antennæ a few times. Enters. Backs out. Flies away. The hole is 5 mm. in diameter.

3 : 38′ 10″. Back with another larva which she takes within. Out, and flies away.

3 : 40′ 15″. *A. porteræ* is seen flying low along ground and entering holes nearby.

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[1] Kindly determined by Mr. S. A. Rohwer.

[2] An account of the habits of this wasp given by C. H. Turner, in BIOL. BULL. *42*, 1922, p. 153–172, 6 figs.

[3] Initial (h) refers to notes of Hicks; (c) to those of Custer.

3 : 41′ 08″. She comes to hole of wasp nest. Enters and remains within.

3 : 43′ 33″. Bee still within. Wasp returns, alighting at edge. *A. porteræ* is near top of hole with jaws spread in a threatening manner, and head blocking the entrance. Wasp jumps at bee in hole, then flies away. Back and strikes at abdomen of bee as the bee backs out of hole. Bee and wasp leave, fighting. Wasp soon returns, alights near hole, tapping it with her antennæ. Enters and is pulling out one larva as *A. porteræ* returns. Bee strikes and tries to bite wasp with her mandibles, a number of times. The wasp flies with larva and drops it 10 inches from hole after which she flies away. Bee then goes inside. Wasp returns but pays no attention to larva which she dropped but comes to hole and looks in for a time. She then flies away. *A. porteræ* leaves.

3 : 54′ 01″. Bee enters hole.

3 : 56′ 52″. Goes away.

4 : 05′ 50″. Bee returns.

4 : 06′. Wasp returns. Tries to enter, jumps back and flies away.

4 : 07′ 02″. Bee flies away.

4 : 18′ 15″. *A. porteræ* enters. Believe carried something inside.

4 : 18′ 40″. Leaves.

4 : 20′ 55″. Bee returns, carrying a load of down.

4 : 21′ 05″. Flies away.

4 : 23′ 40″. Returns with load of down.

4 : 24′ 15″. Leaves.

4 : 26′ 29″. Returns with down.

4 : 27′. Leaves tunnel.

4 : 27′ 01″. Wasp returns. Looks in. Goes in and comes out almost at once. Flies a few inches away. Returns. Looks in. Goes in. Out and is ready to go in again when *A. porteræ* returns with load of down. She strikes wasp and both fly. The bee follows wasp fighting and striking her while in the air. The wasp circles back to the nest followed by bee, and tries to go into the hole. The bee repeatedly strikes wasp as latter tries to enter. *A. porteræ* drives her away, following her out of my view.

4 : 29′ 33″. Neither have come back.

4 : 31′. Wasp back. Looks in and flies away. Back again soon. Looks in. Goes in. Flies away in a circle. Back to nest. In. Out with some down in mandibles. In again and out with down in mandibles. Six more trips in and out with down. Then out dragging larva by head. Gets larva half out and puts back in at once as though she sees mistake. In and out with five more loads of down. Is now 4 : 35′. Comes out again with load of down at which time bee returns flying at wasp and hitting her a number of times. Bee then enters hole. Wasp is back very soon again. Enters hole in which bee is in but backs out at once. Follows an active four minutes in which the wasp repeatedly flies about hole, returns to it, looks in, flies away a few inches, back, etc. The bee is well up the tunnel blocking the entrance, during this period. The wasp flies away.

4 : 41′. *A. porteræ* is still in the hole but has gone down so that cannot see her.

4 : 42′ 55″. Bee out. Flies around and back into tunnel. Goes down into cell.

4 : 46′ 10″. Wasp flying rather high over nest but does not stop.

4 : 48′ 20″. *A. porteræ* out. Flies a few feet away and back carrying nothing.

4 : 53′ 20″. Repeats above.

4 : 55′ 20″. Repeats.

4 : 57′. Bee backs out dragging larva carried in by wasp to the outside. Drops 2 inches from nest. Enters. Out head first. Leaves.

5 : 01′ 02″. Back with down.

From this time until 6 : 30 the bee carried 17 loads of down. The wasp was not seen again. The next day the bee carried 18 loads of down. The day was partly cloudy. On August 4, by 10 o'clock the bee was collecting pollen and by 9 : 15 A.M., August 5, the nest was complete with the tunnel filled with pebbles to the surface of the earth (h).

Anthidium bees have been observed by many students to use down from various plants growing in the vicinity of the nests. Fabre (2) found that some would even readily make use of that from introduced plants. He states that *A. diadema* will take the cotton for its work from any suitable plant growing near its nest. This bee nests in hollow reeds placing as many as ten cells in one stem. The most we have found here have been two in one nest, more usually one. This is due to the fact that most of ours have probably taken over wasp nests providing space for but one or two cells.

According to Friese (3) certain European *Anthidia* obtain their down from the leaves of different hairy plants, among which are especially such plants as *Stachys*, *Ballota*, *Cydonia*, *Verbascum*, *Populus* and *Gnaphalium*.

A. porteræ gathers down from the underside of the leaf of *Cryptanthe gracilis* Osterh.,[1] by scraping it up towards the apex with her mandibles.

After visiting several leaves she then holds the ball gathered with the mandibles between the thorax and front legs and flies with it thus to the nest. A given individual usually returns to the same group of plants to obtain the down. One bee averages two and one-half minutes in securing a load (c).

We have observed a bee to go a distance of over 50 yards in securing the down although some others have been seen to go but 5 yards away while one at White Rocks went but 3 feet from the nest.

In August, 1926, *A. porteræ* was observed to gather down from the stems of live *Artemisia canadensis* Michx.[1] One female was observed while she went from plant to plant visiting a large number on one collecting trip. This bee scraped the down in much the same manner as those observed working on *Cryptanthe*. It would seem that this latter plant is much better for the bee as it has more down and requires less work to obtain it (h).

On July 20 and 21, 1926, notes were taken on the provisioning of a cell, an extraction of which follows (h).

One nest was watched continuously for a number of hours. The opening to the tunnel was slightly enlarged so that the bee could be seen working within. She would return to the nest carrying the down with her mandibles, enter, and arrange it

[1] Kindly identified by Dr. Edna Johnson.

into a cell shaped for the reception of the pollen. She usually turned herself around once and sometimes two or even three times in the placing and working of each load.

In returning to the nest the bee came direct, leaving in the same manner. Although the weather was partially cloudy the bee carried 26 loads of down in two days, an average of six minutes and thirty-eight seconds for each load.

The time of individual trips varied from 4′ 49″ to 16′ 05″. The time spent within the nest, arranging the down varied from 1′ 10″ to 2′ 20″. The time taken to get the down is determined by weather conditions, distance secured from nest, time of day, etc.

At the time of provisioning, the cell had an inner cavity, a little larger than the bee. She began at 1 : 40 to carry pollen and between this time and 3 : 30 carried 6 loads. The time spent within the nest averaged about 2 minutes. On arriving, the bee opened the top of the cell, entered head first, came out, and backed in, forcing the abdomen inside. While here, she removed pollen from the ventral scopa and deposited it in the bottom of the cell. Before leaving the nest, she always partially closed the cell by raking the down together at the orifice. This may make it more difficult for parasites to find or enter the nest.

After depositing the pollen, the bee was not seen to enter the cell again until she had returned from another collecting trip. If nectar was deposited after each collecting trip, it of necessity was deposited first when the bee had her head within. However, the first pollen was seen to be dry and it was not learned if separate trips were made to gather nectar or not. The final food for the young is very moist (h).

After provisioning, laying the egg and closing the cell the bee fills up the tunnel, usually with pebbles. These may be selected from a small area over 9 yards from the nest, in rare instances, but more usually from 1 to 3 yards away. A number of times the bee has availed herself of the pebbles of a large red ant nest (*Pogonomyrmex occidentalis* Cresson).[1] The number of pebbles used is at least enough to fill the tunnel above the cell even with the surface of the ground. The size of the tunnel and the distance above the cell varies. From 135 to 300 pebbles are used, 250 being about the average.

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[1] Kindly determined by Professor T. D. A. Cockerell.

Sometimes a bee loses its hold or attempts to carry a pebble too heavy and is forced to drop it. She then goes at once to the place, from which she secured the one dropped, for another. However in one instance, upon dropping the pebble midway to the nest, the bee alighted at the spot, seized another, and continued on her way. The bee usually drops the pebble from above the hole into the tunnel but on a few occasions she was observed to alight on the edge before dropping it in. Sometimes the pebble fails to hit the hole and remains close to the edge. While filling the hole the bee frequently enters to arrange the pebbles. In doing this she turns them with her mandibles and fits them into position snugly. In only one instance, after the bee had gone inside to arrange the pebbles, have we observed her on leaving to rake in some pebbles and soil from near the edge with her mandibles and fore legs.

On July 24, 1926, found bee filling tunnel. She was watched until the end of this period. The following is an extract from more detailed notes taken in the field (h).

At 8:50 A.M. found *A. porteræ* entering hole in ground with piece of dry dirt. The hole was large enough to see inside and it was noted that only a few pieces had been deposited for the down was not covered. When first observed the bee was carrying material from a place 12 feet east of nest. She carried 10 loads in an average time of 12 seconds. Each time the bee alighted on the ground and selected the piece of dirt. These chunks varied somewhat although the average was quite uniform.

After she carried a few more, a circle of stones was placed about the nest while she was after a load. The stones were about the size of a hen's egg and were 3 inches from hole. On returning she circled 3 times about the hole, then flew 10 feet away, returned, alighted on ground, returned and entered hole. She then went to collect again and continued, returning direct and without further hesitation, paying no more attention to the change. After 15 more loads she changed her place of selecting materials to 18 feet south of nest. The bee, after every few loads, went and arranged them. After 56th trip changed back to position

12 feet east. After 113th trip (9:47) bee went to feed on *Psoralea* plant. She was followed and was found to visit 83 flowers in 6 minutes. She then carried 3 loads into nest and fed again, visiting 15 more. The 139th trip ended at 10 o'clock.

The bee had no sooner alighted, after the 155th trip than a male met her. The period of mating lasted 25 seconds after which the female went on with her work as before. In one instance she carried in a piece of old stem which was so long that it was bent back to get into the hole.

She made in all 240 trips between 8:50 and 10:26 at which time the tunnel was filled even with the surface. This bee carried in almost entirely pieces of dried mud; all others observed have used mainly pebbles. The nest was dug up and consisted of two cells. This nest was of the nature of those made by *Odynerus dorsalis* Fabr. The lower cell was opened and contained a small larva of *A. porteræ* which later died. The upper was not opened until in September at which time a cocoon had been formed containing a live, mature larva. In this instance one mating took place after the egg of the two cells had been laid, both eggs hatching. It is probable that *A. porteræ* mates more than once. Other matings have been observed to take place while the female was collecting pollen for the cell. Once a female of this species was found to be mating with a male, *A. porteræ amabile* Ckll., a variety with more red on the abdomen (h).

The mass of pollen, semi-fluid in consistency, of the cell of *A. porteræ* is 10 mm. long, flat on the top surface and curved on the base. The egg lies flat on top of the mass of provision. A larva 2½ mm. in length was found to be partially embedded in the food. The exact larval period has not been ascertained although a number of cocoons have been formed in the laboratory. The larvæ of those opened never developed much further and it was thought that this might be due to the pollen drying out as well as to possible mechanical injury (h).

The size of the entire cell including the down varies a little, due to the size of hole in which the down is placed. One which was about average in size measured 17 mm. in length, being 12 mm. wide at the bottom and 10 mm. wide at the top. Pellets of excrement mixed with down were all about the down and between the cocoon and the outside (h).

The outer surface of the cocoon has a thin layer of rather light, loosely woven silk. Removing this the denser, darker cocoon is found. An average cocoon measured 10 mm. in length and 5 mm. wide, being oval in shape with the ends somewhat flattened. The anterior end bears a mammillary point which externally contains an opening. This point is not so long nor so conspicuous as the one on the cocoon of *Dianthidium sayi*. One large cocoon measured 12 mm. in length and 7 mm. in width. The cocoons found have been reddish brown. One of the cocoons described, held to a bright light showed the outline of the larva within (h).

We made certain investigations in which we attempted to find if *A. porteræ* would carry pebbles indefinitely to the nest. While the bee was away for a few seconds to obtain another pebble we removed the one, with a pair of forceps, she had last dropped in. Thus the nest was kept continuously empty or partially so.

On July 27, 1926, at Point East at 3:15 P.M. we found a bee carrying large pebbles from the nest of the red ant (*Pogonomyrmex occidentalis* Cress.) 9 yards away. The nest at that time was about half full. We removed 175 pebbles by 5:20 P.M. when the bee quit working for the day. The nest was watched until 7:00 P.M. and from 7:15 A.M. the following morning but the bee was not there at either time and no pebbles had been carried in the interval. She started again to carry pebbles at 7:55 (July 28). From 7:55 to 8:20 she carried 100 stones, which is an average of 4 stones a minute. Later in the day (10:00 A.M.) she was found to be averaging 8 a minute, probably due to the increased intensity and heat of the sunlight. She carried 311 pebbles before noon, having fed early in the morning, suddenly stopped and was seen no more. Although the nest was watched for days, no more pebbles were carried. The total number carried was 741. The bee was not frightened away and apparently quit voluntarily, leaving a number of pebbles in the nest.

To another nest a bee carried at least 400 pebbles and suddenly left, although the hole was kept partly empty as in the other case. Before stopping, in each instance, there was no apparent slowing down of the work, nor cloudy or cool weather. The bee stopped during weather conditions very favorable for work.

In two instances, I have found the nests of some species of *Anthidium* in which the tunnel was unusually short, and over and above which she had carried a mound of pebbles instead of just filling even with the surface, as in the case of nests having longer tunnels. In one instance 135 pebbles and in the other 75 had been carried, more than enough to fill the tunnel (h).

From our investigations it would seem that *A. porteræ* will not carry pebbles indefinitely even under ideal weather conditions.

Whenever the cell becomes damp and mold develops, as sometimes after rain, the nest is deserted.

Both males and females have been found resting in the dry pods of yucca stems during rainy or cloudy weather, in cavities in decayed wood or resting, perched bird-fashion on stems during the night.

During the day when the weather is fine the males dart from flower to flower and are often seen hovering humming-bird fashion in the air. Part of the day is spent in feeding and part in searching for the female. He often darts upon the female while she is working, copulation taking place on the ground. The duration of this period is about 30 seconds. As yet we have reared no parasites.

SUMMARY.

1. No females of *Anthidium porteræ* have ever been found digging a nest.

2. On one occasion she drove away a nesting wasp, *Odynerus dorsalis*, and used the nest for her own.

3. The nests found in which the cells of the bee are placed have in general been such as are made by wasps or other insects nesting at the time.

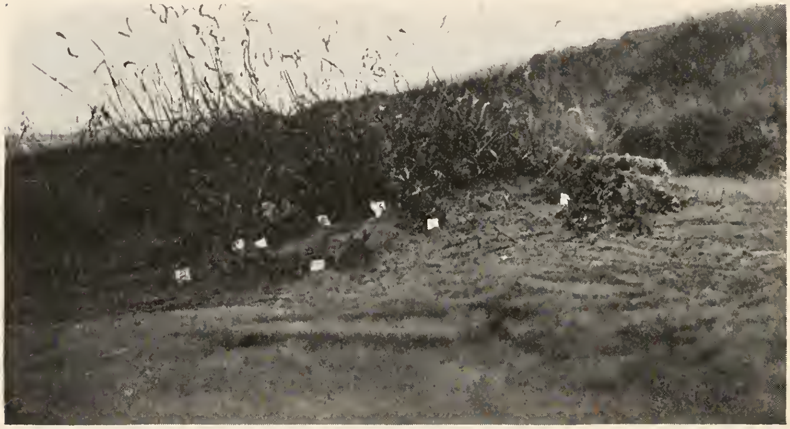
4. *Anthidium porteræ* is solitary and is not found nesting in close association as *Dianthidium sayi* and others.

5. The cell is constructed of down and the tunnel above filled to the surface of the ground, usually with pebbles.

6. When the stones are removed artificially from the tunnel, *Anthidium porteræ* probably will not replace them indefinitely even under ideal weather conditions.

Dianthidium sayi Cockerell syn. *interruptum* Say. (nom. preocc.).

The bees of the genus *Dianthidium* have been known to construct nests of resin on rocks, in stems of plants, in deserted snail shells and other places, but, so far as we know, seldom in the ground as does *Dianthidium sayi.* We have found no reference to the nesting habits of this species except that in 1926 by Hicks (5). This latter information was derived from a number of cells dug up from a small area at White Rocks, during fall of 1925 near Boulder, Colorado. We have obtained our field notes during the summer of 1926 mainly at this locality where two colonies, one hundred yards apart, were found and studied.



The six colonies of *D. sayi,* observed during the latter part of the summers of 1925 and 1926, were all located on hillsides that face south and slightly east. Scattered over these hillsides were tufts of *Bromus brizæformis* Fisch. and Mey.,[1] to the roots of which some of the resin cells were attached. We find that the species may nest as a colony of from eight to fifty or more females, although one apparently solitary female was found nesting in a vertical clay bank. The colony is at its maximum activity during the month of September.

The picture shows a small colony of *D. sayi* on a grass-covered mound of sandy soil one yard in diameter. Base rock surrounds this on all sides, the nearest soil being five feet away. Tall stems of *Bouteloua oligostachya* (Nutt.) Torr. and *Bromus brizæformis* Fisch. and Mey., rise a foot or so above the colony; thus chaff, small sticks, etc., are abundant. The nests were marked, as seen in the picture, 1 to 16 by small numbered strips of paper securely weighted with sand. Nest 7 has been plugged up. The picture shows bee *H*, whose thorax is white for identification, very near and to the left of this nest. She is standing on her hind legs with the fore legs on the bank just above the nest. Nest 4, hidden from view, is a foot behind 2 while nest 14 is a quarter inch above 5. Nests 11, 12, 13, 15 and 16 are hidden from view in the grass to the left of 14. Nests 7 and 8 are about twenty-five inches apart (c).

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[1] Kindly determined by Dr. Francis Ramaley.

All bees concerning which notes were taken were marked with waterproof white paint. Thus the thorax of the bee seen beside label 7 is white. Bee *C*, on whose habits many important notes were taken, was marked as follows: (1) Left half of dorsal surface of last abdominal segment white; (2) A black area present on left side of fourth dorsal abdominal plate completely surrounded by a yellow tegumentary band which area, on right side of same abdominal segment, was not completely surrounded; (3) Right postero-lateral quarter of mesothorax dusty, probably due to some resin sticking there at one time and dust adhering to this (c).

One bee was seen to have two nests in the process of construction at which she worked more or less alternately. Later, five such bees were observed. These were shown to and verified by Hicks. The following short extract illustrates this habit in the case of one bee: Bee *C* is constructing two nests which are nine inches apart. Into one nest she carries pebbles, lumps of dirt, chaff and such sticks as she can handle with her mandibles. Then she flies directly to the other nest and does the same thing there. Thus she carries the following number of these articles

To Nest 1:	Then to Nest 2:
6	6
4	5
9	0

(Then a male arrives and they mate while she has a stick in her mandibles (60 seconds). He leaves and she continues her work after carrying this stick to nest 1.)

To Nest 1:	Then to Nest 2:
23	0

(She flies to flowers of *Grindelia* several yards away, and feeds there for ten minutes; then she returns to work as before.)

To Nest 1:	Then to Nest 2:
5	1
5	0

Here she flies to the fields. One of the sticks is one and one half inches long (c).

The following account shows this habit in another individual: Bee *D* goes into nest 3 for fifty-five seconds. After going out and in several times, she leaves, flies slowly over to nest 4, nine inches away, and goes in at once. Presently she leaves this nest and flies to some nearby flowers to feed. In one minute she arrives at nest 3, leaves after a few seconds, and flies slowly over to nest 4 which she enters. After one half minute, she leaves, feeds for five minutes and goes into nest 3. She comes out, three quarters of a minute later, goes into 4 and spends one half minute there before leaving for the fields to feed (c).

It has not been observed whether or not *D. sayi* digs the hole for its nest and so it can not be proved that one bee has attended to two holes from the first. However, the proximity of the nests to one another indicates that it does the former. It might easily be that each summer, when the colony began to nest, there was but one bee to each hole. Then when some of the builders died, before completing their nests, others of the colony would appropriate these deserted nests and finish them along with their own. The following examples are taken from the notes to give evidence that temporarily deserted nests of *D. sayi* are readily taken over by other members of the colony:

Example 1.

Sept. 14, 1926. While bee *I*, the owner of nest 8, is in the field, another member of the colony comes and goes into the nest. Thirty-five seconds later, bee *I* arrives and goes into nest 8 also. Presently the owner backs out and is followed closely by the intruder. The former alights on the intruder's thorax, biting with the mandibles at the junction of head and thorax. A short, lively tussle follows, after which the intruder escapes and the owner goes on working.

Example 2.

Sept. 17, 1926. Bee *D*, which has been constructing nests 3 and 4, has now spent ten minutes carrying chaff, etc., into 4. In the meantime, bee *I*, which has been working on nest 8, arrives from one of her numerous trips to the field and goes into nest 3. She at first makes regular trips carrying soil out of this nest. Then she seems to be carrying pollen in. The owner of nest 8 has been working on 3 all day and has but rarely visited 8. The owner of nests 3 and 4 has continued to carry chaff, etc., solely into 4, eventually plugging it up. The next day the owner of nest 3 returns. The owner of nest 8 sees her return and so is now working as before on 8 (c).

Five bees by this time (Sept. 17) have been seen repeatedly to visit two nests apiece. To summarize, they are: (1) Bee *C* to nests 1 and 2; (2) Bee *D* to nests 3 and 4; (3) Bee *E* to nests 5 and 14. (4) Bee *I* to nests 8 and 3 (to 3 only to-day); (5) Bee *H* to nests 6 and 7. There are eight bees working in the colony. Of the sixteen nests, nine are open and seven are plugged, but of the latter, numbers 7, 15 and 16 are not being worked upon by the bees. Thus the eight bees of the colony are working on thirteen nests, and three of these bees are seen to work on but one nest apiece (c).

On September 26, we dug up the colony and found 151 completed cells from the 16 nests, which is an average of 9.44 cells to a nest. This was practically the end of the nesting season, for at this time and later the colonies of *D. sayi* were deserted.

It is interesting to note, from a standpoint of comparative entomology, that a wasp, *Sphex varipes* (Cress.)[1] has been observed to be taking care of two nests also (h).

From these facts and others in our study of bees, we have found that there is sometimes considerable variation in the habits of different individuals of a given species.

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[1] Kindly determined by Mr. S. A. Rohwer.

The picture shows bee *H* in one of her numerous visits to the plugged hole 7. Her action suggests that a bee retains the memory of her nest for at least a week after it has been completely plugged up and that her visits are made to it to insure its security from enemies. A brief extraction from my field notes illustrates her action: "Bee *H* goes into nest 6 for one and one half minutes, comes out and goes over to plugged hole 7. She spends about ten seconds here. Upon arriving at 7, she first puts her head down against the plug, rubs it with face and mandibles and scratches weakly with fore legs for three or four seconds. She then stands erect before the nest on her hind legs with her fore legs against the bank above the plugged hole (see picture). After standing thus motionless for about five seconds, she flies away to the fields. She repeats this procedure many times daily in like manner. Only occasionally does she first go to nest 7 and then to 6" (c).

While bringing out soil from within the nest, after the tunnel has been constructed, bee *D* has been observed to back out, raking it with her forelegs. She usually leaves this within an inch of the entrance although in a few instances she took it out in this manner as far as six inches, and once ten inches (c).

D. sayi obtains the resin used in the construction of the nest from the small sunflower, *Helianthus petiolaris*. She obtains this resin, with her mandibles, from the stem and partially or totally dried leaves which have small droplets and plates of it distributed over them (c).

Fabre (2) studied "resin workers" for many years but states that he never saw a bee get resin. Friese (3) states, in reference to the source of resin used by a species found in Europe, that it is probably obtained from the buds of the pine. Hacker (4) inferred that *Megachile rhodura* used resin for its nest since he observed members of both sexes visiting a *Eucalyptus* tree, from which resin had oozed, and rasping the resin with their mandibles. However, he did not find *M. rhodura* nesting. Melander (7) presumes that the resin used by *Anthidium texanum* Cresson was obtained from cedar. Hungerford and Williams (6) say, in their account of *Dianthidium concinnum* (?) Cresson: "The nest of this insect is composed of pebbles glued together with resinous cement which may be derived from the stems of *Helianthus* which are often infested with a small lepidopterous borer that causes an exudation which usually attracts a host of Hymenoptera."

The following field notes were taken, giving the resin activities of bee *C* during one hour:

12:57½. Arrives with resin in mandibles at nest 2. Kneads resin with mandibles.

1:00. Obtains a piece of chaff an inch away from nest and inserts it into the resin which by now she has shaped into a plug over the ends of the pieces of chaff projecting from the burrow.

1:06¾. Carries piece of chaff to nest 2.

1:07. Obtains small stick and flies with it to nest 1.

1:07½. Flies away. I follow her. She goes to some flowers of *Grindelia* and feeds.

1:09½. Flies to some dried plants of *Helianthus petiolaris*. Picks a plant without many red and black ants present and scrapes resin off with mandibles. She visits three plants.

1:12¾. Arrives from fields at nest 2. Has in her mandibles a load of resin about one fourth as large as head. Distributes resin over ends of chaff projecting from the entrance.

1:21. Flies to nest 1.

1:21½. Leaves nest 1. Goes directly to sunflower plants and secures resin.

1:24. Arrives at nest 2.

1:25½. Leaves nest 2.

1:30. Arrives and leaves after two seconds. I follow her to several flowers of *Grindelia* where she feeds.

1:32. Arrives at nest 2 and leaves, after two seconds, for the fields.

1:34½. Arrives at nest 1 with white resin in mandibles. Works here with the resin in the construction of a plug.

1:36¾. Obtains large pebble two inches away and inserts it into resin of nest 1.

1:38. Flies to nest 2 and works with plug there.

1:42. Carries stick to plug at nest 2.

1:42½. Leaves nest 2 and feeds at flowers of *Grindelia* and of *Helianthus petiolaris*.

1:46. Arrives at nest 2. Leaves after two seconds and again feeds.

1:48. Arrives at nest 2. Walks all over colony looking into several nests and returns to this one.

1:49. Flies away.

1:51. Arrives with resin at nest 2.

1:51½. Obtains piece of chaff and takes to nest 1. Gets stick midway between nests 1 and 2 and carries it to nest 2.

2:01. Leaves nest 2 and obtains resin after feeding on flowers of *Grindelia*, etc. (c).

The entrance to the tunnel, after the completion of the cells, is closed by a plug of resin, chaff and other materials. From several plugs taken out, we find that the resinous part is about 2 mm. thick. Usually, in constructing the plug, the bee first places a few pieces of chaff, sticks, etc., in the entrance and then builds a resin cap over the projecting ends. Beyond the plug, towards the cells, the tunnel is often empty and may be coated with resin.

One of us (Hicks) (5) has described the cells of *D. sayi*. Last summer it was not known whether all the materials found in the resin had been carried there by the bee or were there by chance. Observations this summer show that she obtains the chaff, sticks, etc., which are found mixed with this resin, from the vicinity of the nest. In some instances she has been found to carry fifty or more pieces of chaff, etc., into one tunnel until it was filled. After this she finished the nest with a resin plug. The resin cells are often found attached to the grass roots in similar fashion to peanuts on the stem.

D. sayi varies considerably in the distance which she carries the stones, dried mud, etc., with which she constructs the nest. Bee *J* (attending to nest 9) is seen to make many trips with plates of dried mud from an old mud puddle, six yards away, to her nest. Bee *E* (attending to nests 5 and 14) is seen to carry many large pebbles to her nest from a sand pile eleven yards away. She places these in nest 14 after having plugged up 5 (c).

D. sayi may also obtain chaff and sticks from a distance varying from three inches to three yards away. One individual selected material near the nest but did not repeatedly obtain it from the same place; another repeatedly visited a more distant source. This latter was usually found to reject several pieces of chaff, etc., before taking one to the nest (h).

The female of this species (bee *C*) was seen to mate twice within three days. The first mating, on September 13, is recorded in field-notes given on page 270. The second mating is given in the following extraction: On September 16, bee *C* leaves nest 1, which is now almost totally plugged up with chaff, etc., and flies to some flowers of *Grindelia* to feed. She then flies to some small sunflowers nearby and feeds there also. Finally she goes to a flower of *Lygodesmia juncea*. While feeding there, a male arrives and they mate. This mating takes sixty-five seconds. The female is grasping the stamens of the flower with the fore and mid legs and is touching the hind legs repeatedly on those of the male. This is apparently the first record of a wild bee mating more than once (c).

The male has been observed to await at the colony the return of a female. As soon as she arrives, he darts at her to effect copulation and in some cases they collide in mid-air, both falling to the ground. The female, with partially outstretched wings, rests motionless except for a slight movement of the hind legs on those of the male. The male has the female grasped around the third abdominal segment with the forelegs and beneath the abdomen with mid and hind legs while the tips of his antennæ are bent forwards and outwards. His only movement is a relaxation and contraction of the abdominal segments and a slight waving of the tips of the antennæ. The average time for copulation, from seven examples, is very close to a minute (59.7 sec.). We have observed that mating may occur both at the colony and at the flower while the female is feeding. Although the male is absent from the colony most of the time, nevertheless the majority of the matings observed have taken place at the nest.

Mating probably does not interfere with the nest-building activities as evidenced by the field notes on the first mating of bee *C*. Furthermore, a female has been seen, returning laden with pollen, to mate, after which she immediately went into the cell, deposited the pollen and went on with her work.

D. sayi has been observed to feed on the flowers of the following plants:

(1) *Grindelia squarrosa.*
(2) *Helianthus petiolaris.*[1]
(3) *Sideranthus spinulosus.*[1]
(4) *Lygodesmia juncea.*[1]

After a rain, which partially filled the entrances of some holes, we completed the closing of these with wet sand to observe the later action of the owners. One, entrapped within the nest, used the mandibles to remove the plug; another, returning from the fields, also used her mandibles to gain entrance. The latter was observed to place head and mandibles into a small excavation, which she had formed, and to walk in a clockwise direction around the hole with the tip of the abdomen describing a circle. The forelegs were practically useless in this process. The abdomen was not used in this case to pack the soil in repairing the walls, as has been observed in a bee of the genus *Augochlora* under similar circumstances.

D. sayi has not been found feeding beyond a radius of one hundred yards from the nest. However, of six marked females released four hundred and fifty-five yards from the colony, four returned within five hours. The shortest time required to return this distance was twenty-one minutes. Two specimens released one hundred yards from the nest returned shortly, the first within fifteen minutes, after feeding along the way (c).

The only parasite of *D. sayi* which has been bred is a fly of the genus *Villa* (Anthrax),[1] Hicks (5). Some mutillids and chrysids may be parasitic since they have been frequently observed to enter the open or partially plugged-up nests.

SUMMARY.

1. Several instances of a female bee, *Dianthidium sayi*, working alternately on two nests in the process of construction, have been found. This has also been observed in a species of wasp, *Sphex varipes*. Apparently these are the first records of this habit.

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[1] Determined by Professor T. D. A. Cockerell.

2. Resin, used in the construction of the nest, is obtained from the leaves and stem of the small sunflower, *Helianthus petiolaris*.

3. *D. sayi* is one of those wild bees whose members nest in close association with one another.

4. A female of this species was seen to mate more than once.

5. *D. sayi* seems to be nearly free from parasitism.

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ORIGIN OF THE MESODERM AND BEHAVIOR OF THE NUCLEOLUS IN REGENERATION IN *LUMBRICULUS*.[1]

LEONARD P. SAYLES.

Introduction.

The microdrilous annelids have been the center of considerable interest on the part of students of regeneration particularly from the point of view of the origin of the cells which form the new tissue. While the production of new endodermal and ectodermal structures from cells of the corresponding old parts is generally accepted, the origin of the mesoderm in the bud has been a point of contention. Hepke ('97) in Naids, von Wagner ('00 and '06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, are of the opinion that the new mesodermal structures both in anterior and in posterior regeneration are of ectodermal origin. Randolph ('92) in posterior regeneration in *Lumbriculus* reports their formation from comparatively unmodified cells of the old mesoderm to which she gives the name "neoblasts." Iwanow ('03) in *Lumbriculus* and Krecker ('10) in *Tubifex* and *Limnodrilus* verify her conclusions. They find that in anterior regeneration, however, cells from old specialized mesodermal structures form the new portion. One of the features in the descriptions of the cells of the bud region by these workers is the prevalence of large nucleoli in the cells involved in the regenerative activity. Krecker ('23) gives a rather complete description of the origin and migration of the neoblasts in posterior regeneration. Studies on the origin of the new mesoderm in regeneration together with observations on nucleolar changes in these and other tissues are reported in this paper. For invaluable advice on this work I am indebted to Dr. J. W. Wilson of Brown University at whose suggestion the problem was first undertaken.

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[1] This paper forms a part of a thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Brown University, June, 1926.

Materials and Methods.

The species used in this work was identified for me by Professor Frank Smith, of the University of Illinois, as of the genus *Lumbriculus*, and probably the species *inconstans* Smith. Anæsthetization was avoided as far as possible. When it was necessary, a one eighth of one per cent. solution of chloretone or a four per cent. solution of alcohol, redistilled in glass, gave equally satisfactory results. For histological fixation Zenker's fluid or Bouin's Picroformol were used; best results were obtained with the latter. It was not necessary to anæsthetize the worms first. An individual to be fixed was placed on a glass plate and straightened against a glass slide. Another slide was then pushed up until it came in contact with the worm, care being taken not to crush it between the slides. In this process only a very small amount of water was used. The fixing fluid was then slowly added and its surface tension, as it was drawn underneath the two slides, held the worm firmly in place during the preliminary fixation. This method is a modification of one described by Welch ('13). As a stain Heidenhain's iron alum-hematoxylin method was used for the most part, though preparations were also made with Delafield's hematoxylin and eosin, used both as a direct and as a regressive stain. On carefully controlled regressive staining the chromatin granules were stained with the hematoxylin while the nucleoli took the eosin, indicating that they were true nucleoli or plasmosomes.

Nuclei and Nucleoli in the Cells of Uninjured Individuals.

As this paper is to deal at some length with the cytological changes, particularly nuclear and nucleolar, which occur during regeneration, it is perhaps advisable first of all to discuss more or less completely the conditions which exist in uninjured individuals. Under this heading the usual appearance of cells in the hypodermis, setigerous glands and intestine, including those differences which may exist at the growing region of the tail, will be described.

Hypodermis.

In all the epithelial cells of the hypodermis the nuclei are quite large, all gradations from an oval type (the average measurement of twenty-five of which is 3.3 x 5.8 μ), to the practically spherical one (averaging about 4.2 μ) are to be found. In each a very minute nucleolus, appearing as a mere dot under a magnification of 1,000 ×, is visible (Fig. 1). Cells of this type form all of the hypodermis from the second segment to the growing region of the tail. At this end, just anterior to the place where the basement membrane is discontinuous due to the formation of the ventral nerve cord, the hypodermal cells are slightly elongated. The nucleoli are slightly larger than in the cells located more anteriorly (Fig. 2). Cells of this type are probably intermediate stages between the active ones of the growing region and the typical hypodermal cells found in the older part of the body. Such an elongation of the cells becomes even more marked where the nerve cord is forming with the result that the hypodermis in the growing tip is two or three times as thick as in the rest of the body wall. This elongation of the cells is most marked ventrally just posterior to the end of the nerve cord. The thickening of the hypodermis includes the dorsal and lateral portions as well as the ventral but it does not extend as far anteriorly on the dorsal side as elsewhere.

Bülow ('83), in his discussion of the normal growing region, describes this thickening, but makes no mention of any difference in the nucleoli of this region. In posterior regeneration, however, Krecker ('10) describes a marked enlargement of the nuclei and nucleoli of the ventral ectoderm. In the normal growing worm the nuclei are larger near the growing nerve cord and a short distance posterior to it than they are elsewhere (Fig. 2); the average size of these nuclei is 3.9 x 7.4 μ. The cells in which such a difference is apparent are the ones involved in the formation of the new nerve cord. The nucleoli, on the other hand, are enlarged throughout the entire tip and to a slight degree for a short distance from it. This difference is most decided in the ventral cells, in which the nucleoli may be nearly 2 μ in diameter, but average between 1 and $1\frac{1}{2}$ μ.

Setigerous Glands.

Each pair of setæ is embedded in a mass of cells derived from the ectoderm and to them are attached secondarily mesodermal cells which form their musculature (Penners, '23). The setæ themselves are formed by the ectoderm cells, according to the description of Bergh ('90), more recently corroborated by Penners. In the earlier stages of the formation of the setigerous glands in the growing region of the tail most of the cells possess oval nuclei of about the same size as those of the neighboring ectoderm cells and nucleoli measuring between 1 and $1\frac{1}{2}$ μ. As muscle fibers are added to the outside of the gland cells it becomes more difficult to study the latter but it seems certain that most of them have large nucleoli for a number of segments from the growing region. In the older part of the worm, however, the nucleoli are smaller, for the most part mere dots (Fig. 3). Occasionally a few cells are to be found possessing fairly large nucleoli (1 μ); these cells may have been active in the formation of a new seta to replace one which had been lost.

Alimentary Canal.

The cells of the alimentary canal in the first segment are typical hypodermal cells, of cuboidal shape with round nuclei 4 to 5 μ in diameter and nucleoli which appear as mere dots (Fig. 1). These cells, unlike those of the rest of the digestive tract, are without cilia but covered with a cuticle, as are those of the hypodermis (Pointer, '11). In the second somite the cells are more elongated and the nuclei are of the oval shape (3.6 x 6.9 μ) typical of most gut cells of this species. This segment is a transition zone between the first and third as far as the nucleoli are concerned. In the latter the nucleoli are fully $\frac{1}{2}$ μ in diameter (Fig. 4).

These same measurements hold for both the nuclei and the nucleoli in the cells of the next few segments. At about the ninth or tenth segment, however, cells with larger nucleoli appear. These cells increase in abundance until in the vicinity of the thirteenth practically every nucleolus measures at least 1 μ (Fig. 5). There is no corresponding enlargement of nuclei. A feature which first appears in this region is the occurrence of two nucleoli within a single nucleus. This is a phenomenon which is rather infrequent in this species except in regenerating individuals. For brevity, they will be spoken of as "double nucleoli" but this meaning of the words should not be confused with that of earlier workers, particularly Montgomery ('98), whose "double nucleoli" were individual nucleoli made up of two types of material. While no unquestionable cases of double nucleoli have been observed in the first eleven segments, posterior to this region, where the nucleoli are larger, cases of this sort are occasionally found. Counts made on several uninjured individuals indicate that the average frequency is about 3 per segment.

This larger type of nucleolus is typical of the gut cells for a considerable distance. Then there is a gradual decrease in size. In some individuals it seems to be at considerable distance from the anal segment—even 30 segments or more—while in others the larger nucleoli apparently persist at least 15 or 20 segments farther. In this posterior portion of the gut they are hardly more than mere dots in the nuclei (Fig. 2).

Mesoblasts.

In the ventral portion of the cœlom in the growing tail region, cells are present which are presumably derived from the mesoblasts of the developing individual (Wilson, '89 and '92). These cells, which produce the mesodermal structures in the newly forming segments, possess large nuclei (about 4 x 8 μ) and nucleoli (about 2 μ) (Fig. 2).

Large nucleoli are present, therefore, in the cells of the middle portion of the gut, in those of the hypodermis of the growing tail region and in the mesoblasts. The largest of these are in the cells of the ventral region of the ectoderm, which are forming the new nerve cord, and in the mesoblasts, from which the new mesodermal structures are derived. The significance of these facts will be discussed under a later heading.

Origin of New Tissue in Posterior Regeneration.

Endoderm.

In posterior regeneration in microdrilous annelids the majority of investigators, especially more recently, are agreed on the method of origin of the endodermal structures. Krecker ('10) in *Tubifex* and *Limnodrilus* removes a portion of the intestine after cutting a worm and finds that by a proliferation of cells in the old intestine growth takes place until it comes in contact with the ectoderm. He says (p. 411) that "mitosis and amitosis both occur," using as a criterion for the latter "the frequent occurrence of double and elongating nucleoli." He finds that cell division may take place one or two segments anterior to the wound, but when it "was seen at such a distance it was always found to be mitotic . . . ." His observations are verifications of statements to the same effect made by Iwanow ('03). A study of individuals which have been regenerating for various periods reveals that mitoses may and commonly do occur even eleven or twelve segments from the wound region. The distribution of mitotic figures and double nucleoli in worms regenerating for different periods is given in Tables I. to VI., inclusive.

Table I.

1 Day of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	8	8	7	7	10	6	4	4	5	3	2	2	2	3
Mitoses	0	0	0	0	1	0	0	0	0	0	0	0	0	0

Table II.

2 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	25	31	20	25	21	25	23	20	22	23	19	18	6	2
Mitoses	10	8	4	3	7	4	3	3	2	5	2	2	0	0

Table III.

3 Days of Regeneration.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	21	34	25	27	23	25	21	23	17	9	9	7	3
Mitoses	14	2	5	2	5	3	3	0	2	5	3	1	1	0

TABLE IV.

4 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	26	26	53	46	32	19	15	8	8	7	6	3	1	2
Mitoses	5	6	4	7	6	1	0	0	1	1	1	1	0	0

TABLE V.

5 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	44	37	41	33	24	18	18	9	9	6	5	6	3	2
Mitoses	6	5	7	3	4	1	4	3	1	1	0	0	0	0

TABLE VI.

6 DAYS OF REGENERATION.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli	20	23	18	17	20	15	16	7	5	3	4	2	1	3
Mitoses	3	2	4	1	1	0	1	0	0	0	1	0	0	0

In making these counts it is necessary to adopt some criterion for double nucleoli. These figures, therefore, are for cases where a nucleus contains two nucleoli in approximately the same focal plane of the microscope. In order that the personal equation involved in making the counts may be made as negligible as possible, instances of dumbbell-shaped or elongated nucleoli are not included. Records are not given for segments beyond the fourteenth from the wound since but 2 or 3 double nucleoli per segment, the number ordinarily found in an uninjured worm, are to be found in these. For mitotic figures all stages from the appearance of the chromosomes in the prophase to the late telophase are included.

As already mentioned, the gut cells of these worms ordinarily have nucleoli of considerable size, averaging about 1 μ in diameter, and the oval nuclei measure on the average about 4 x 7 μ. With the onset of regeneration an increase in the size of the nuclei and nucleoli begins, though it is comparatively slight in the former case.

Twelve hours after a worm is cut such a change in the nucleoli near the wound is apparent and by the second day many of them measure at least 2 μ in diameter (Fig. 6). Similarly by the second day the nuclei have enlarged until the average of a number measured is 5.1 x 8.8 μ. This increase in the amount of nuclear and nucleolar materials is not confined merely to the wound region but extends even eleven or twelve segments from it. Farther away than this, however, these structures show no change from the size ordinarily present in such a region (Fig. 5).

Double nucleoli are found in increased numbers within the same limits as are enlarged ones and beyond the twelfth or thirteenth segment from the wound they are no more abundant than in an uninjured individual (Tables I. to VI. inclusive). There is no gradual decrease in number, however, as one gets farther from the wound but a somewhat abrupt drop about the twelfth segment. Mitoses, too, are found only in these same twelve or thirteen segments and, though usually most numerous in the two or three segments nearest the bud, they too do not gradually decrease in number but stop rather abruptly.

Table VII.

Number of Double Nucleoli.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	40	26	66
2	122	150	272
3	133	127	260
4	183	66	249
5	179	71	250
6	98	52	150

Double nucleoli and mitoses increase rapidly in numbers during the early part of the regeneration period and then gradually decrease again. Double nucleoli have begun to appear on the first day but only one instance of mitosis is found. There is a great increase in the number of each on the second day. By the fourth day, however, the number of double nucleoli has dropped decidedly in the segments more distant from the wound; this is counterbalanced by an increase in the five nearest segments with the result that the total number is not appreciably changed. Table VII. gives the comparative abundance of double nucleoli in the first five segments and in the other seven which are apparently involved in the regenerative processes.

The number of mitoses also decreases in the more distant portion but in this case it is not compensated by an increase near the wound so that the total number is less. The major part of the activity of the gut in forming new tissue, therefore, is now confined to a more restricted area. Table VIII. gives data for mitoses corresponding to that given in Table VII. for double nucleoli.

TABLE VIII.

NUMBER OF MITOSES.

Number of Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
1	1	0	1
2	32	21	53
3	28	17	45
4	28	5	33
5	25	10	35
6	11	2	13

The records for the fifth day are approximately the same as those for the fourth. On the sixth the numbers both of double nucleoli and of mitoses have decreased decidedly throughout the entire region involved in regeneration. This decrease is most marked, however, in the more distant segments. By the seventh day the intestine of the old region has returned to approximately its usual appearance in an uninjured worm. Only two or three cases of mitosis are to be found and double nucleoli are little if any more abundant than in intact animals.

Beginning with the second or third day many cells in which the nucleoli are small are present in the intestine of the old part. These are probably the products of a recent mitotic division during which the nucleolus has been lost. That the nucleoli of these cells enlarge gradually is evidenced by the fact that the number of such cells does not increase appreciably during further regeneration. Similarly most of the cells in the newly formed gut tissue of the regenerating bud have small nucleoli at first.

By the third day these, too, are enlarging so that it is not possible to discover a line of demarcation between the gut of the bud and that of the old part. At about this time double nucleoli and mitotic figures begin to appear in the new gut. Cell proliferation in the new tissue thus increases as that in the old part is decreasing. Consequently, as far as the intestine is concerned, regeneration usually ceases between the sixth and seventh days. After this time it is a matter of growth in the newly formed tissue itself.

Mesoderm.

The method of formation of the new mesoderm is not as generally agreed upon as is that of the new intestine. Hepke ('97) in Naids, von Wagner ('06) in *Lumbriculus* and Abel ('02) in *Tubifex* and *Nais*, among others, maintain that the new mesodermal structures originate from ectodermal cells which migrate into the cœlom. Randolph ('92) and Iwanow ('03) in different species of *Lumbriculus* and Krecker ('10 and '23) in *Tubifex* and *Limnodrilus* find that these structures are derived from relatively unmodified cells of the mesoderm to which the term neoblasts was first applied by Randolph. Krecker ('23) reports that they are ordinarily found on the posterior surfaces of the septa in a quiescent state. After a worm is cut they enlarge to 8 or 10 times their former size and then migrate along the nerve cord to the wound region. According to his data these cells are activated on the seven septa nearest the wound with those in the four nearest segments giving the greatest response. In my preparations of individuals which have been regenerating for either three or four days a number of clearly distinguishable neoblasts are always present as far as eight or nine segments from the wound. Two examples, one of a three-, the other of a four-day regenerate, are given in Table IX. All the cells which lie upon the septa and are clearly neoblasts with nucleoli of 2 μ or over are included in these counts. All are found on the posterior surfaces of the septa and are confined almost entirely to the ventral portion. These cells correspond presumably to the later stages of metamorphosis described by Krecker ('23). In both of these cases nine segments are clearly contributing neoblasts while the tenth and eleventh are apparently not involved. It is a rather common occurrence to find one or two neoblasts on a septum in an uninjured worm so that the few seen in these last segments are in accordance with what is to be expected. In making these counts, not only leucocytes, as suggested by Krecker ('23), but also nephridial cells are apt to be mistaken for neoblasts in early stages of metamorphosis. Cells of this type, as clearly demonstrated by examination of adjacent sections, are shown in Fig. 9. Both of these types of cells have quite large nuclei and nucleoli and many nephridial cells are of course in contact with the septa.

Table IX.

Neoblasts.

Segments from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	17	13	16	13	9	6	7	3	6	1	1
4-day regenerate	15	19	18	14	8	7	8	8	7	2	1

On the four or five septa nearest the wound, therefore, neoblasts are most abundant, as also observed by Krecker ('23). About nine segments in all, however, seem to be involved while Krecker reports the maximum distance as seven. The region involved in the production of neoblasts is, therefore, approximately the same as that in which cell proliferation occurs in the intestine.

Ectoderm.

The ectoderm, unlike the other two types of tissue already discussed, regenerates solely from cells in the immediate vicinity of the wound. That cells from the old hypodermis form the new hypodermis and nerve cord is generally agreed upon. Krecker ('10, p. 430) describes the marked enlargement of certain ectoderm cells "not directly opposite the nerve but somewhat dorsally, between it and the central longitudinal axis of the body." These cells increase in size and become so changed that, as he expresses it (p. 434), "were these cells seen alone they would be immediately considered neoblasts." Of the changes which take place in cells other than those of this particular region, however, he makes no more than the simple statement (p. 430) that "of course the ectoderm cells are greatly enlarged elsewhere than on the ventral side."

Twelve hours after a worm is cut the wound has healed and in those ectoderm cells which are brought into a terminal position by this process the nucleoli have begun to enlarge (Fig. 10). The cells themselves are beginning to elongate somewhat and accompanying this process the nuclei are changing to the oval shape more or less typical of elongated cells. The nuclei themselves are not appreciably enlarged at this time but the nucleoli have increased from mere dots to quite evident structures about 1 μ in diameter. Cells of this type are not confined to any definite part but make up the entire terminal portion of the hypodermis. After one day of regeneration the enlargement has gone still further but there is no marked difference in size between cells in the ventral portion and those dorsally located. The average size of the nuclei at this time is 4.5 x 7.2 μ and of the nucleoli 1.4 μ.

On the second day, however, characteristic differences make their appearance (Fig. 11). The cells which are dorsal or lateral show little if any change from the preceding day. In the median ventral region just posterior to the end of the nerve cord many of the cells have grown considerably but all gradations in size can be found between the largest of these and the cells found elsewhere in the bud. In the larger cells the nucleoli are oval and about one third the dimensions of the nuclei. The average size of ten of the larger nuclei found in two adjacent sections is 6.1 x 8.4 μ, with the nucleoli of the same cells averaging 1.9 x 2.8 μ. On the following day the nuclei of the dorsal cells are of about the same size as on the second day but many of the nucleoli have enlarged to 2 μ. In the ventral region the changes are even more marked. A number of the cells have enlarged enormously and have nuclei of about 8 x 11 μ, with nucleoli averaging about 3.5 μ. It is this stage of the hypodermal cells to which Krecker applies the term "metamorphosed" ectoderm and which may be most easily confused with neoblasts. Krecker suggests that the fact that the neoblasts come to lie near such cells is probably the reason why so many workers have thought the neoblasts to be products of the ectoderm, which seems very plausible.

Krecker ('10, p. 436) suggests, as a theory of the formation of these much enlarged cells, that "the neoblasts have a redifferentiating effect upon the cells of the ectoderm." A close examination of the facts, however, demonstrates that the cases which he cites in proof may all be explained in another way.

In the course of regeneration the enlargement of the hypodermal cells does not appear to begin suddenly soon after the arrival of neoblasts at the wound. It seems rather to be a continuous process having its inception at the time the worm is cut. A certain time is required for the cells of the hypodermis to enlarge and those on the ventral side do not change any more rapidly than those elsewhere. They do, however, show a greater response than the others with the result that their enlargement continues after the others have slowed down. In sections of twelve-hour regenerates the ectoderm is quite different from that in uninjured individuals and the process of enlargement is clearly under way. At this time neoblasts are rarely found at the wound. Krecker ('10, p. 422) says that "twelve hours after the operation . . . in one of these (individuals) there was a neoblast at the wound, but none was migrating. In the other individual . . . two were about the wound." If it is true, then, that the enlargement of the ectoderm begins before twelve hours after the cut is made, and it certainly appears to, it is improbable that the neoblasts could have been the cause. A much simpler explanation, which seems to satisfy all of the requirements of the facts at hand, is that whatever is the underlying cause of the metamorphosis of the neoblasts on the septa is likewise the cause of the enlargement of the cells of the hypodermis at the wound. Just what may cause the neoblasts to metamorphose is not discussed by Krecker.

This view that the transformation of the ectoderm cells is independent of the presence of the neoblasts is entirely in accord with the cases cited by Krecker ('10) in proof of his theory. In one individual (p. 433) "even after three days there was no enlargement of the ectoderm. No neoblasts were about the ectoderm, in fact there was only one neoblast to be seen and this was along the nerve some distance away." He says later (p. 436) that this "exception cited in which no change in the character of the ectoderm cells occurred in the absence of the neoblasts is of considerable significance." This case may be equally well explained in accordance with the view of an independent transformation. The factor which brings about the metamorphosis of the neoblasts and ectoderm is either absent or, because of the physiological condition of this particular individual, produces no effect. It is reasonable to suppose that, if the same underlying cause brings about like changes in these two types of cells and if they are equally susceptible to activation, one type will not undergo a process of transformation when the other does not. In all other cases given by Krecker for posterior regenerates he himself states (p. 435) that "the evidence adduced has to do only with instances in which neoblasts are found about the metamorphosed ectoderm cells." Admittedly neoblasts are present in all cases where the ectoderm cells are greatly enlarged, but an explanation for this is easily found. Neoblasts migrate only along the ventral nerve cord and so they are found in the ventral region of the bud. The ectoderm cells which are greatly enlarged are those which are to give rise to the nerve cord in the ventral region. The close proximity of these two types of cells in the regenerating bud seems to be due to the fact that both bear a definite relation to the nerve cord—those of the hypodermis to form the new portion of the nerve cord in the bud and the neoblasts using it as a pathway to the wound region. In the discussion of anterior regeneration his statement that no metamorphosis of the ectoderm and no migration of the neoblasts occurs in this type of regeneration will be considered.

The development of the setigerous glands and the production of the setæ have for the most part been neglected in the work on regeneration. Bergh ('90) and Penners ('23) both give something of a description of their formation during embryonic development. In regeneration it is difficult to observe the early stages due to the great number of cells scattered throughout the growing bud. By the fifth day the cells of the glands in the two or three segments of the bud nearest the old tissue stand out clearly since at this time the other cells of these segments have become arranged in a more orderly fashion. The nuclei of the hypodermal cells near the old tissue have begun to take on the appearance typical of the older cells. They are oval—measuring about 3.5 x 6 μ—and contain nucleoli less than 1 μ in diameter. The nuclei of these gland cells, however, have not decreased in size but are about the same as those of the enlarged ectoderm cells of the dorsal and lateral regions (Fig. 12). The average measurement of twenty-five of these is 5.2 x 7.3 μ with nucleoli 1.8 μ in diameter. Occasionally double nucleoli are to be found. The fact that these cells push into the cœlom offers another point of confusion which may have led early workers to think that the ectoderm migrated into the cœlom to form the new mesodermal structures.

It will be seen that in all three types of cells (endodermal, mesodermal and ectodermal) which take part in the formation of new tissue at the posterior end there is one outstanding feature in common. The nuclei and nucleoli enlarge. The greatest changes occur in the neoblasts and in the cells of the ventral portion of the hypodermis. In both of these types the nuclei of the fully transformed cells are eight or nine times as large as ordinarily. The nucleoli increase even more in proportion, particularly in the hypodermal cells in which they enlarge from mere dots to structures over 3 μ in diameter. Even in the gut cells where the nucleoli are usually of considerable size there is probably a ten-fold increase. The significance of these facts will be discussed later.

Origin of New Tissue in Anterior Regeneration.

Endoderm.

In anterior regeneration as in posterior regeneration the majority of investigators—Rievel ('96), Haase ('98), von Wagner ('00), Iwanow ('03) and Krecker ('10)—are agreed that most of the intestine of the regenerating bud is formed by the growth of that in the old part. This formation of new tissue involves about eleven or twelve segments here as in posterior regeneration. The nucleoli are considerably enlarged at the end of the first day and double nucleoli are fairly common. Only a few mitoses, however, are present. The numbers both of double nucleoli and of mitoses reach a maximum between the second and third days. On the fourth day both are fewer in number in the more distant segments. In the case of double nucleoli this decrease is partially compensated by an increase in the region nearest the wound. Mitoses, however, are somewhat less frequent in this region too. As in posterior regeneration the numbers continue to decrease and by the seventh day both of these features are rare in the gut of the old part. Tables X. and XI. give records for two- and four-day regenerates, respectively. Tables XII. and XIII. give comparative data for the nearer and more distant segments for double nucleoli and mitoses, respectively.

There is little, if any difference, then, in the behavior of the cells of the intestine in anterior and in posterior regeneration.

TABLE X.

2 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.	27	26	21	27	34	30	30	33	32	29	16	16	1	1
Mitoses.	12	6	3	7	8	4	3	4	2	3	2	0	1	0

TABLE XI.

4 DAYS OF REGENERATION.

Segments from the wound.	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Double nucleoli.	38	31	29	32	34	26	14	7	8	6	2	3	3	1
Mitoses.	12	4	3	4	7	3	1	0	1	1	0	0	1	0

TABLE XII.

DOUBLE NUCLEOLI.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	135	186	321
4	164	66	230

TABLE XIII.

MITOSES.

Days of Regeneration.	Segments from Wound.		Total.
	1–5 (incl.).	6–12 (incl.).	
2	36	18	54
4	30	7	37

Mesoderm.

Many investigators—Hepke ('97), von Wagner ('00) and Abel ('02) among others—are of the opinion that the mesoderm both of anterior and of posterior regenerates is formed from the ectoderm. The weight of evidence in posterior regeneration, as previously mentioned, now seems to favor mesoderm formation from neoblasts. In anterior regeneration Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* find that the new mesoderm is formed from the old mesoderm and not from regeneration cells as in posterior regeneration. They find a distinct fraying out of the longitudinal muscles at the wound. Many of the cells then lose their contractile substance and wander about in the cœlom where they become mixed with other cells—peritoneal, connective tissue and ectodermal—so that it is "hard to distinguish between the various types" (Krecker, '10, p. 441). After about the fifth day of regeneration they begin to develop contractile substance and to become arranged in muscle masses. The production of the body musculature is much slower in anterior than in posterior regeneration. "In specimens killed three weeks after the operation the musculature of this region was still in a very undeveloped state" (Krecker, '10, p. 442).

As the matter stands at present, then, investigators are divided into two groups regarding the formation of the new mesoderm. Those in one believe that it is derived from the ectoderm, those in the other that it is produced by a partial dedifferentiation followed by a redifferentiation of the cells of the old mesodermal structures. Those who support an ectodermal origin of the mesodermal structures believe that the mesoderm is formed in the same manner at both ends. Those of the other group, however, think that the origin is due to one type of mesodermal cell at the posterior end—the neoblasts—and to another at the anterior end—the specialized cells which dedifferentiate.

In anterior regeneration, just as in posterior regeneration, there are many opportunities for confusion. In the former it is even more difficult than in the latter to follow just what happens. The migration of ectoderm cells into the cœlom in the formation of the cerebral ganglion might easily mislead one. In addition to this the presence of many other cells of similar appearance make it practically impossible to say, from an examination of fixed specimens, just what cells do form the various tissues. Since in posterior regeneration it is quite apparent that ectoderm is not involved in the formation of the septa or longitudinal muscles it seems reasonable to assume that it does not take part here. This view is supported further by the fact that cells of mesodermal origin are present in the bud.

Regarding the conception that there is a partial dedifferentiation of the old muscle cells which then form the longitudinal musculature of the bud, there seems to be conflicting evidence. These cells certainly do fray out and some of them seem to lose their contractile substance in the segment injured by the cut. Such a behavior is not, however, peculiar to the anterior end in *Lumbriculus inconstans*. Just as other cells are affected by the cut so many muscle cells are dislodged or injured. It is not improbable that such cells should then lose their contractile substance due to the injury; such a change might, therefore, be a step on the road to destruction rather than on that to repair. The fact that this same sort of change does take place at a posterior cut-surface, where the muscle cells are not involved in the regenerative processes, seems to support this view. The migrating spindle-shaped cells, which appear between the first and second days in *Lumbriculus*, are clearly derived from the hypodermis (Fig. 13). These cells migrate into the cœlom and there form the cerebral ganglion. It may perhaps be this type of cell which has been observed by Krecker in *Tubifex* and *Limnodrilus*.

Further evidence is derived from a study of neoblasts in anterior regenerates. Krecker finds that in the species with which he worked they are usually not activated at all posterior to a cut and that resting neoblasts may be found on a nearby septum. "The individuals upon which these observations were made were all killed three weeks or more after the operation so that the failure of the neoblasts to act as they do at the posterior end could hardly have been due to lack of time" (Krecker, '10, p. 437). In the individuals used in his experiments—of the genera *Tubifex* and *Limnodrilus*—anterior regeneration does not take place at the level of the twentieth somite where these cuts were made. It does not seem strange, therefore, that at the end of three weeks all neoblasts should be resting even if activation had taken place three weeks previous. In *Lumbriculus* anterior regeneration does take place at all except very posterior levels. The amount of tissue is, of course, limited to a few segments, usually five or six, rarely seven (von Wagner, '00).

Counts made of the number of clearly recognizable neoblasts (intermediate and definitive stages of Krecker, '23) present on the various septa in three- and four-day regenerates are given in Table XIV. These cells all possess nucleoli of approximately 2 μ or over (Fig. 14).

Table XIV.

Neoblasts.

Segment from the wound	1	2	3	4	5	6	7	8	9	10	11
3-day regenerate	6	21	14	9	11	13	3	3	4	2	1
4-day regenerate	16	14	23	13	7	8	2	6	3	1	1

These figures do not differ greatly from those for posterior regenerates (Table IX.). The small number of neoblasts present on the first septum of the three-day regenerate may be explained in part by the fact that this septum was partially torn away by the cut in this particular case. About four segments seem to produce neoblasts more abundantly than the rest and about nine segments in all are apparently involved. The more active region extends farther, however, in the three-day regenerate; the inclusion of the first septum in the cut brings this about. The neoblasts, then, are activated to about the same extent as at the posterior end. The number present in the bud at the end of two days, however, indicates that either not as many migrate anteriorly or else the migration is much slower and the cells are used up as they get to the wound. No mass of large cells is to be found in the ventral part but several cells are usually present and a number can generally be found migrating along the nerve cord (Fig. 15). The apparently slow migration of the neoblasts anteriorly is perhaps the reason why the mesoderm in the newly formed head does not show signs of development until after the fifth day (Krecker, '10). After the third day it is difficult to say just what does happen in the bud region. Many metamorphosed ectoderm cells are present and things are so confused that it is difficult to distinguish the various types of cells, particularly those as similar in appearance as are the neoblasts and the much enlarged cells from the ventral hypodermis.

The process of formation of new septa and longitudinal muscles by cells known to be derived from neoblasts has not been observed. The fact that neoblasts metamorphose in considerable numbers and are frequently seen migrating along the nerve cord is, nevertheless, evidence which seems to indicate that they play some part in anterior regeneration in *Lumbriculus*. If such is the case, the mesodermal structures are formed in the same manner in both types of regeneration, just as are the ectoderm and endoderm.

Ectoderm.

As in posterior regeneration only those cells in the immediate vicinity of the wound take part in the formation of the new ectodermal structures. There occurs a similar enlargement of all these cells during the first day or so, no one region undergoing any more extensive change than any other. Iwanow ('03) in his discussion of the formation of the new nerve elements at the anterior end describes a marked enlargement of the ectoderm cells. Krecker ('10, p. 433) says that the ectoderm cells "undergo no such metamorphosis" as at the posterior end, explaining this on a basis that no neoblasts migrate anteriorly to stimulate it to activity. As in the case of posterior regeneration, however, by the second day there are cells on the ventral side which have quite large nuclei and nucleoli, and by the third day they have reached a considerable size (Fig. 16). The area over which this marked enlargement extends is greater than in posterior regeneration. Large nuclei are present in cells found over a considerable portion of the ventral and ventro-lateral hypodermis and even somewhat dorsally in the terminal portion. The largest cells, nevertheless, are confined for the most part to the ectoderm in the vicinity of the mid-ventral line. This region extends from the end of the old nerve cord anteriorly. The nuclei and nucleoli measure about the same as in the transformed cells of the regenerating tail (8 x 11 μ and 3.5 μ, respectively). In the dorsal portion of the terminal hypodermis many of the cells are apparently in the process of breaking away from the epithelium to migrate into the cœlom where they form the cerebral ganglion (Fig. 13). They become elongated and slender in this process and develop an appearance which answers well the description of cells which Krecker ('10, p. 441) believes are mesoderm cells migrating anteriorly to form the new longitudinal muscles—"spindle-shaped cells with slightly granular cytoplasm and large nucleus containing a deeply staining nucleolus."

Regarding the activation of the ectoderm by the presence of the neoblasts, there is no further evidence from anterior regeneration. Neoblasts apparently migrate anteriorly and the ectoderm is also transformed, similar processes to those occurring at the posterior end.

From these observations it is apparent that not only the ectodermal and endodermal elements of the regenerated head are derived in the same manner as at the posterior end but the mesodermal structures as well. Just as in posterior regeneration the neoblasts apparently metamorphose and migrate to the wound region and the cells of the ventral portion of the hypodermis become greatly enlarged. About ten or eleven segments seem to be involved in the regenerative processes except in the case of the ectoderm in which the changes are confined to the immediate vicinity of the wound as in posterior regeneration.

Discussion.

The production of new tissue in the anterior regeneration of microdrilous annelids seems to be essentially the same as in the posterior regeneration in these forms. Iwanow ('03) in *Lumbriculus variegatus* and Krecker ('10) in *Tubifex* and *Limnodrilus* describe the formation of the new mesodermal tissue from neoblasts at the posterior end but believe that there is a dedifferentiation of old mesoderm to form the new at the anterior end. The loss of contractile substance by some of the longitudinal muscle cells at the cut surface, however, occurs at both ends. The spindle-shaped cells abundant in the dorsal part of the bud in anterior regeneration may be seen in *Lumbriculus inconstans* to be derived from the hypodermis, migrating into the cœlom to form the cerebral ganglion (p. 295). The fact that many neoblasts metamorphose and migrate anteriorly is evidence that they probably take part in the building of the new mesoderm in anterior as well as in posterior regeneration. The presence in the bud of other cells of similar appearance makes it practically impossible to follow the laying down of these structures from cells of known origin. It has for some time been agreed by investigators that both endodermal and ectodermal structures are formed in the same manner in both types of regeneration, that is, from the old intestine and body wall, respectively. It seems evident then that the mesodermal structures are produced in the same way at both ends and are not an exception as previously believed.

An explanation for the formation of the new mesoderm from more or less undifferentiated mesodermal cells rather than from the muscles may perhaps be found in the fact that the cytoplasm of the muscle cells is highly modified. While the cells of the hypodermis and intestine are of a simple, cuboidal or columnar shape, the development of the contractile substance by the muscle cells brings about an extensive modification of the cytoplasm of these cells. Consequently, cells from the peritoneum, less modified than the others are called upon to form the tissue in the regenerating bud. The development of the new nerve cord is a process not essentially different. In this case the cytoplasm is considerably modified in the formation of fibers and, instead of the nerve cord near the wound dedifferentiating to produce the new tissue, the hypodermal cells of the ventral side are called upon to furnish the new material. While these facts may not necessarily indicate that one type of cell is any "more differentiated" than another, the muscle and nerve cells in *Lumbriculus* certainly are less susceptible to the activating stimulus than are those of the other tissues.

In regeneration the ectoderm cells enlarge considerably especially those in the median ventral line which may develop nuclei as large as 8 x 11 μ with nucleoli between 3 and 4 μ in diameter. Krecker ('10 and '23) is of the opinion that this change in the ventral cells is due to "some influence" of the neoblasts. Evidence presented here (pp. 290 and 297) does not support this idea but rather the view that a similar underlying cause brings about the changes which occur in the metamorphosis both of the neoblasts and of the ectoderm cells. During the first two days of regeneration all the cells of the hypodermis in the immediate vicinity of the wound develop large nuclei and nucleoli. After this time the ones found dorsally and laterally slow down whereas those in the ventral region continue to enlarge. Neoblasts are rarely, if ever, found at the wound at the time when the increase in size of the hypodermal cells becomes apparent (between six and twelve hours after the cut). From that time until the largest cells are formed the enlargement seems to be a continuous process without any sudden change or increase in rate which might be produced by the presence of neoblasts. The fact that in posterior regeneration metamorphosed ectoderm cells and neoblasts are always found very near one another is cited by Krecker ('10) as proof that there is some relation between them. This proximity of these two types of cells is perhaps equally well explained if the reason for the presence of each in the ventral region is considered. The cells of the ventral hypodermis are transformed in the process of proliferation and migration to form the nerve cord of the regenerating region. The neoblasts migrate to the posterior end from the old tissue along this same structure. These cells, then, are brought together not because one causes the modification of the other, but rather from the fact that each type has a certain relation to the nerve cord. The view that the same cause produces the changes in the cells of both types seems to fulfill all the requirements and to be a simpler explanation than that given by Krecker. Furthermore, there is no apparent reason why the ectoderm, any more than the endoderm, should need to be activated by another type of cell.

Function of the Nucleolus.

Various theories have been advanced regarding the function of the true nucleolus or plasmosome. Montgomery ('98) and Ludford ('22) give a rather complete discussion of the work of many investigators. The more recent results seem to indicate that the nucleolus bears some relation to the activity of the cell body and nucleus. Many cases have been reported of nucleolar extrusions preceding the formation of yolk granules. Ludford ('22) also reports that in the more active cells of the endoderm of *Limnæa* the nucleoli are larger than in those of the hypodermis. He is "inclined, therefore, to regard the size of the nucleolus as an indication of the degree of metabolism existing in the cells—the greater the metabolic activity, the larger the total volume of nuclear (nucleolar ?) matter present in the nucleus, or extruded into the cytoplasm" (p. 139). Wilson ('25, p. 96) also believes that there is a "question whether the nucleolus may not play a more active and important part in cell metabolism than most writers have hitherto assumed."

An examination of slides of *Lumbriculus*, both of uninjured and of regenerating individuals reveals no cases of nucleoli which could be interpreted as being extruded into the cytoplasm from the nucleus. There is considerable evidence, however, that nucleolar size is in some manner an indication of the degree of metabolic activity of the cell.

In uninjured worms the gut cells of the first eleven or twelve segments have small nucleoli. Similarly those for some distance from the anal opening have a comparatively small amount of nucleolar substance. In the intermediate portion, however, relatively large nucleoli are present. The cells of the mouth cavity and esophagus naturally do not take as great a part in the digestive processes as do those found more posteriorly. In the mid-gut the digestive fluids are being poured into the lumen and the food is being absorbed. Consequently considerable cell activity is necessary. Toward the posterior end such activity naturally drops off again. The size of the nucleoli, therefore, parallels more or less the extent of the activity expected of the cells in the various regions of the gut (p. 281).

Furthermore, in the case of the setigerous glands, the nucleoli of the cells are large in the growing tail region and in a regenerating bud where the new setæ are being rapidly formed (pp. 281 and 291). In the old segments of a worm, however, where the setæ have been present for a considerable time, the nucleoli are usually small (p. 281). The cells in the active portion of the nephridia possess nucleoli of considerable size, too. In fact, these cells are very similar in appearance to intermediate neoblasts, from which they may be distinguished by the presence of large granules in their cytoplasm (p. 288). Similarly, as described by Krecker ('23), the phagocytes have very large nucleoli.

Again, in the growing tail, just as in the regenerating individual, the hypodermal cells have enlarged nucleoli, particularly on the ventral side where the nerve cord is being formed (p. 280). Those cells which are forming the new mesoderm—presumably derived from the primary mesoblasts in the embryonic development (Wilson, '89 and '92)—also have very large nucleoli, differing very little in appearance from the neoblasts in a regenerating tail (Randolph, '92). In contrast to this, in the older part of the worm, the nucleoli of the mesoderm and ectoderm cells are very small, in the case of the latter mere dots under a magnification of 1,000 ×.

In regeneration, as already mentioned, the nucleoli of the neoblasts and hypodermal cells become greatly enlarged. There is also at this time an increase in the amount of nucleolar substance in the gut cells. For ten or eleven segments from the wound, the nucleoli enlarge, a process followed by the appearance of numerous instances of double nucleoli—two within a single nucleus (p. 285).

This occurrence of double nucleoli is taken by Iwanow ('03) and Krecker ('10) as evidence that amitosis is frequent in the production of the new gut tissue. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli. The individual nucleoli in the case of the double one are usually smaller, and in no case larger, than those in the neighboring cells where but a single nucleolus is present. It seems, then, that the division of the nucleolar material into two parts is not in preparation for a succeeding cell division. Rather as this material accumulates it continues to exist in a single droplet until it reaches a certain size and then divides. This splitting into two parts may perhaps be due to the fact that a droplet of material of its consistency and composition has a certain maximum size beyond which it cannot exist as an individual droplet under the conditions existing in the nucleus. This view is in accord with our knowledge of the limitations to drop size in emulsions.

After one day of regeneration many cells show this increase in the amount of nucleolar material, as evidenced by the occurrence of a number of double nucleoli as well as the enlargement of the single ones. On the second and third days the frequency of double nucleoli reaches a maximum and after that time falls off slowly. Following the increase in nucleolar material, there appear numerous cases of mitosis. It seems probable that this increase in nucleolar substance is indicative of a heightened activity on the part of the cells in preparation for cell division.

A comparison of the amount of nucleolar material in the various types of cells taking part in regeneration reveals a distinct parallelism between this amount and the relative activities of these cells. There are four general types of cells involved: (1) those of the old gut which form the new gut; (2) those of the old dorsal and lateral hypodermis which build the new hypodermis; (3) those of the old ventral hypodermis of which the special function is to furnish the material for the nerve cord in the regenerating bud; finally (4) the neoblasts which form the new mesodermal structures. Of these, the cells of the first two types maintain to a certain extent their usual epithelial arrangement, only a comparatively small amount of migratory activity occurring. Their nucleoli enlarge considerably but by no means as much as in the case of the other two types. The ventral ectoderm cells and the neoblasts, when fully transformed, are of about the same general size and appearance and have nucleoli of nearly twice the diameter of those in the other cells. The ventral ectoderm cells must naturally undergo rapid proliferation to supply all of the material necessary for the nerve cord; the neoblasts must migrate to the wound region and there multiply with considerable rapidity. There is some relation in *Lumbriculus*, then, between the functional activity of the cells and the amount of nucleolar material present in them. This is a conclusion similar to that drawn by Ludford ('22) from a study of *Limnæa* with particular reference to the behavior of the nucleolus in oögenesis and cleavage.

SUMMARY.

Nuclei and Nucleoli in Uninjured Individuals.

1. The nuclei and nucleoli of the hypodermal cells are small except in the growing tail region. Here they are enlarged, especially in the cells on the ventral side which are involved in the formation of the new nerve cord.

2. Large nucleoli are present in the cells of the setigerous glands near the growing region of the tail. In old segments they are small.

3. The gut nucleoli are small in the first twelve segments. They are larger from this region up to twenty or thirty segments from the posterior end. In these segments, they are again small.

4. Double nucleoli are occasionally found in the mid-gut, where large nucleoli are present.

Origin of New Tissue in Regeneration.

5. Double nucleoli and mitoses are found in the intestine for eleven or twelve segments from the wound. In this same region the nucleoli are considerably enlarged.

6. Cell proliferation in the old intestine practically ceases between the sixth and seventh days of regeneration.

7. Neoblasts metamorphose and migrate to the wound at the anterior end as well as at the posterior end. At least eight or nine segments furnish these cells, the four or five nearest the wound apparently playing the most important part as observed by Krecker.

8. The failure of the muscle and nerve cells of the old part to form the corresponding new structures in regeneration is perhaps due to the fact that the cytoplasm of these cells has become highly modified, thus rendering them less susceptible to activation.

9. The spindle-shaped cells in the dorsal portion of the bud cavity at the anterior end are derived from the hypodermis and not from the muscles of the old part.

10. In both anterior and posterior regeneration the nuclei and nucleoli increase in size in the ectoderm cells in the immediate

vicinity of the wound. This enlargement is no more rapid in one part than in another; it continues longer in the ventral cells so that by the second day it is greater there.

11. The metamorphosis of the ectoderm is not in all probability due to the proximity of the neoblasts, as supposed by Krecker, but instead to an independent transformation.

12. The cells of the setigerous glands in the new bud have large nuclei and nucleoli.

13. A feature common to all the cells which take part in the formation of the tissues in the regenerating bud is the presence of large nuclei and nucleoli.

14. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division.

15. In *Lumbriculus* there is no evidence of any division or even of a clearly defined constriction in any of the nuclei of the gut which contain two nucleoli.

16. The presence of two nucleoli in a single nucleus is not a step in amitosis, as many have supposed, but is due to the increase in nucleolar substance beyond the amount which can exist within that particular nucleus as a single droplet.

17. The various tissues seem to be derived in the same manner both in anterior and in posterior regeneration.

CONCLUSIONS.

1. Both in anterior and in posterior regeneration the mesoderm is formed from neoblasts. Iwanow and Krecker are in error in the belief that cells from old specialized mesodermal structures form the new ones in the anterior regeneration of *Lumbriculus*.

2. There is a certain predetermined area of the hypodermis on the ventral side which metamorphoses preparatory to the formation of new nervous tissue during regeneration. The cells of this region are probably activated by the same stimulus as are the neoblasts. Krecker's view that the neoblasts have an inciting effect on the cells of this region seems unfounded.

3. The amount of nucleolar material present in a cell seems to be an index of the activity of its nucleus both in cell-metabolism and in preparation for cell division. Two nucleoli within a single

nucleus are the result of an increase in nucleolar substance beyond the amount which can exist within the nucleus as a single droplet. Their presence is not a step in amitosis.

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KEY TO PLATES.

All figures are photomicrographs. With the exception of Fig. 11, all were taken with a Zeiss apochr. 2 mm., eyepiece 10; these are magnified 640 ×. For Fig. 11, a B. and L. apochr. 4 mm. and eyepiece 6 were used; this is magnified 290 ×. The following are the symbols used in the figures:

a., anal opening,
c., cuticle,
chl., chloragogue cells,
d. e., enlarged dorsal ectoderm cells,
d. n., double nucleoli,
g., gut,
gl., gland,
h., hypodermis,
m., mitosis,
m. c., mouth cavity,
m. e., metamorphosed ectoderm cells,
mes., mesoblast,
migr., migrating ectoderm cell,
n., nucleus containing nucleolus,
neph., nephridial cell,
nb., neoblast,
nv., nerve cord,
s., seta,
sep., septum.

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PLATE I.

Uninjured Individuals.

FIG. 1. Mouth cavity and hypodermis, showing small nuclei and nucleoli in this region.

FIG. 2. Growing region at posterior end.

FIG. 3. Setigerous gland of an old segment.

FIG. 4. Gut of fourth segment.

FIG. 5. Gut of thirteenth segment.

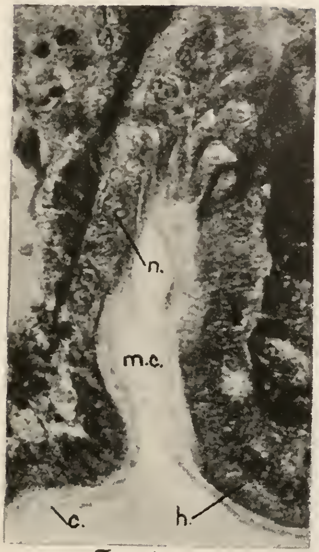
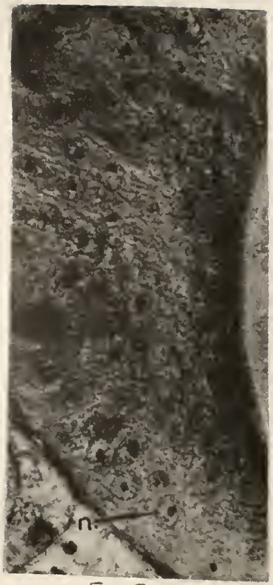


Fig. 1.



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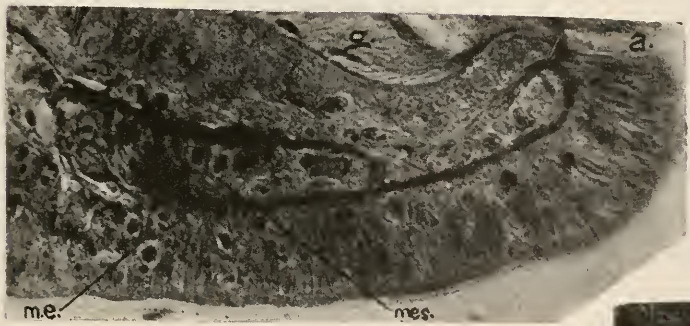

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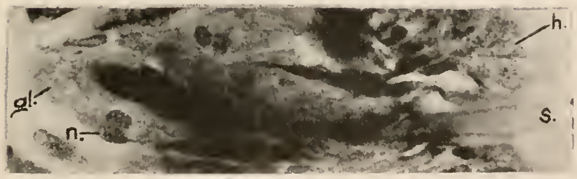

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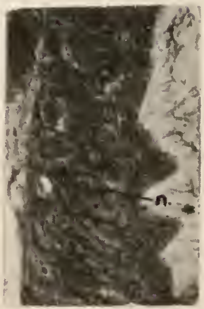

Fig. 4.

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Plate II.

Regenerates.

Fig. 6. Two-day posterior regenerate. Gut cells of second segment from wound.

Fig. 7. Same individual as in Fig. 6. Gut cells of tenth segment from wound.

Fig. 8. Same individual as in Fig. 6. Double nucleoli in gut cells of fourth segment from the wound.

Fig. 9. Three-day anterior regenerate. Nephridial cells in a position in which they might be mistaken for neoblasts. Arrow points toward the anterior end.

Fig. 10. Twelve-hour posterior regenerate. Enlarging ectodermal cells in wound region.

Fig. 11. Posterior regenerating bud at the end of two days.

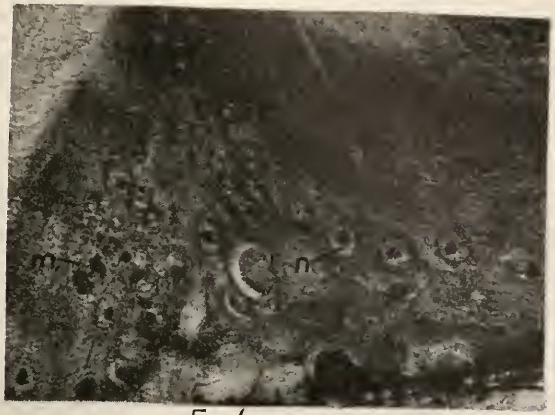


Fig. 6

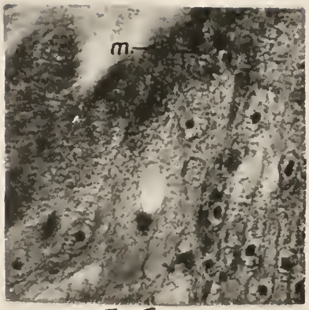
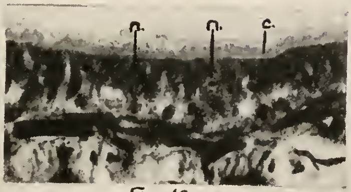


Fig. 7



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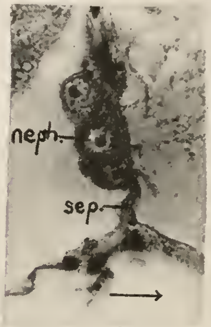

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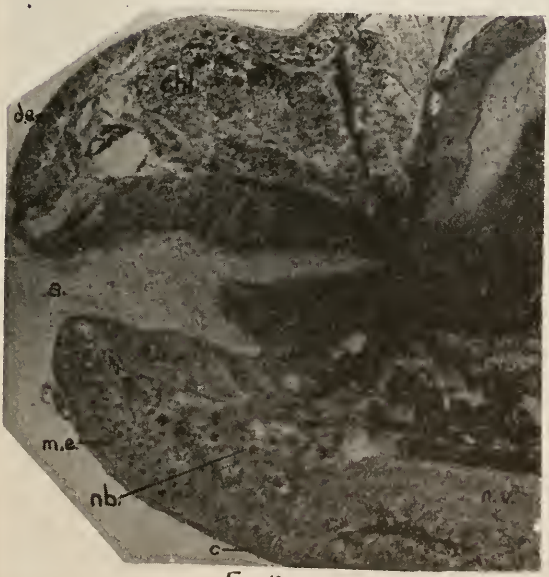
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Fig. 11.

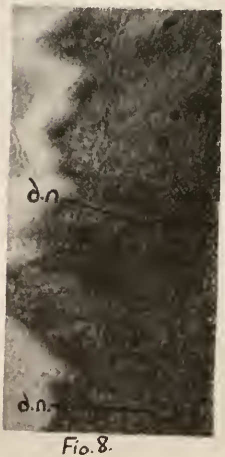

Fig. 8.

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Plate III.

Regenerates.

Fig. 12. Six-day posterior regenerate. Setigerous glands producing new setæ.

Fig. 13. Two-day anterior regenerate. Spindle-shaped cells of ectoderm migrating into bud cavity.

Fig. 14. Two-day anterior regenerate. Neoblasts metamorphosing on the posterior surface of the fifth septum from the wound. Arrow points toward anterior end.

Fig. 15. Two-day anterior regenerate. Neoblasts at anterior end of nerve cord.

Fig. 16. Three-day anterior regenerate. Metamorphosed cells of ventral ectoderm.

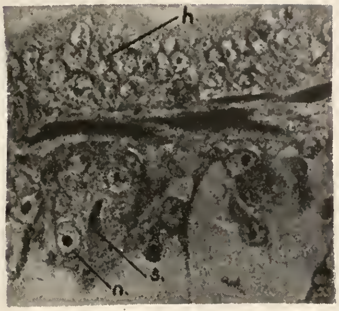

Fig. 12.

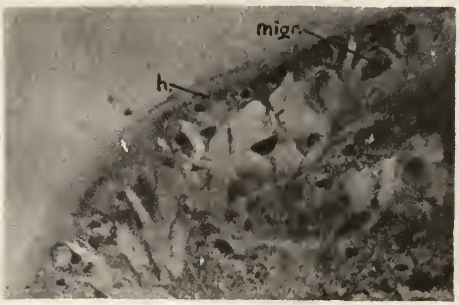

Fig. 13.

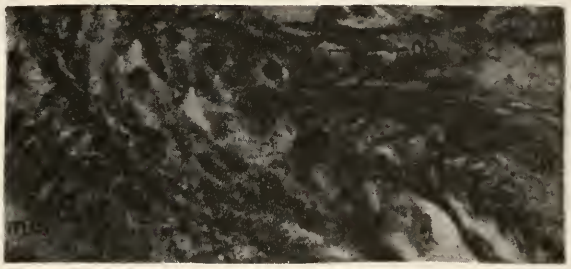

Fig. 15.

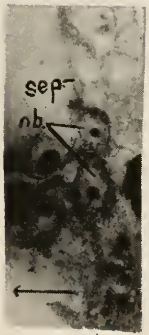

Fig. 14.

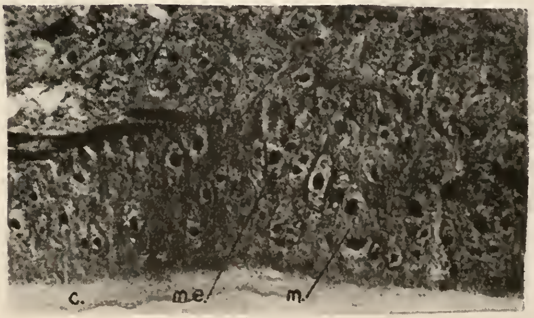

Fig. 16.

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BIOLOGICAL BULLETIN

THE EFFECT OF CYANIDES ON THE SWELLING OF PROTOPLASM.

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Physiologists have long since attempted to formulate a theory for the cause of anæsthesia, and the results thereof have been many and varied. The exceptions to any one theory are too numerous and too important to accept any one of them as the correct theory. The final and real solution of so complex a physiological process will probably be one which will take into consideration portions of all theories now existing, or one which will introduce an entirely new factor or factors. Accounts of the various existing theories are set forth in a number of papers among which are the following: Overton ('01), Meyer ('99), Traube ('19), Warburg ('14), Mathews ('14), and Lillie ('18). This paper will make no attempt to give any evidence in support of any one of the theories, but will rather present the results obtained in using HCN and KCN as anæsthetics.

The work here reported deals with the effects of HCN and KCN on the permeability of unfertilized *Arbacia* eggs to water. An increase in the volume of the eggs, when placed in a solution, is taken as showing an increase in the permeability of the egg. Heilbrunn ('25), however, objects to calling an increase in the volume of the egg an increase in permeability. He suggests that this increase in size may be due to a decrease in surface tension; an increase in the fluidity of the interior or an increase in the extensibility of the plasma membrane. Since *Arbacia* eggs are nearly all spherical, changes in their volume may be measured by measuring the changes in the diameters of the eggs with an ocular micrometer. Eggs from a single individual were placed in concentrations of HCN varying from *N*/300 to *N*/2,000. At the end of ten minutes and again after 25 and 66 minutes, eggs were pipetted into a 50 per cent. solution of sea-water (hypotonic) and their diameters measured at regular time intervals. A control was kept in which sea-water replaced the HCN solutions.

The HCN solutions were prepared by drawing over, by means of an aspirator, into a wash bottle containing distilled water, the volatile HCN gas from another bottle containing KCN to which a few drops of acid had been added. The amount of HCN going into solution was determined by titrating with 0.1 *N* $AgNO_3$; 1.0 cc. of the nitrate being equal to 0.013 gram HCN. It was necessary to use distilled water because the chlorides in the sea-water interfered with the titration. The desired concentrations of HCN were then made up with sea-water. All the sea-water mentioned in these experiments was sea-water from which CO_2, in excess of that in equilibrium with the CO_2 in the air, had been removed. This was done by adding 2.4 cc. of 0.1 *N* HCl per liter of sea-water and aerating for 18 to 24 hours. NaOH or HCl was then added to bring it to the pH of normal sea-water.

The eggs, after exposure to the cyanide solutions, were placed in syracuse watch-glasses containing the hypotonic solution and a 4 mm. objective used as a water immersion with a 10× ocular. This gave a magnification of 450×. Readings were taken one minute after placing in the hypotonic solution and thereafter every minute for ten or fifteen minutes. A stop watch was used to read time intervals. The following abstract taken from daily notes will show the routine followed throughout the experiments.

7/20/26. Temperature of room 21° C. pH of HCN solution measured colorimetrically =7.2. *9A.M.* unfertilized *Arbacia* eggs placed in 30 cc. of *N*/300 HCN in a finger bowl and covered. *9.10 A.M.* eggs pipetted into 5 cc. hypotonic sea-water in watch glass and diameter of eggs measured every minute for ten minutes. Three perfectly spherical eggs were measured each time and the average taken. *9.25 A.M.* some eggs from original *N*/300 HCN solution placed in hypotonic solution and ten one-minute readings again taken. Temperature of room 21.2° C. *10.06 A.M.*, after exposure of 66 minutes to HCN solution, ten one minute readings again taken. Temperature of room 21.7° C.

The 10, 25 and 66 minute readings were repeated using solutions of HCN up to *N*/2,000. Similar readings were also taken using sea-water instead of HCN solutions. These served as controls. Each time that a new sea-urchin was used, the diameters of the eggs were first measured to be sure that they were approximately the same size as eggs which had been previously used.

As previously stated, the HCN was drawn over into distilled water and then diluted with sea-water. The original HCN solution (with distilled water) usually reached its saturation point as an *N*/130 HCN solution. In order to bring this to an *N*/300 HCN, an amount of sea-water almost equal to the original amount of distilled water had to be added. This in itself, therefore, was a 57 per cent. solution of sea-water and the change in volume of the eggs might very well be due to that hypotonicity instead of to the effect of the HCN. This was checked by adding to the control solutions of sea-water, as much distilled water as was contained in the various concentrations of HCN. That is, the control for the *N*/300 HCN was a 57 per cent. sea-water solution; for the *N*/500 HCN a 74 per cent. sea-water and for the *N*/2,000 HCN a 93 per cent. sea-water solution. The results with these various controls showed that only in the high dilutions, *i.e.*, 57 per cent., 67 per cent. and 74 per cent. sea-water solutions, did this hypotonicity have any appreciable effect on the volume of the eggs, but even this increase was much less than the increase in volume of the eggs previously exposed to the HCN solution. The dotted curve in Fig. 1 marked Control A is the control in 100 per cent. sea-water, while the curve marked Control B is the control in 67 per cent sea-water (comparable to the *N*/400 HCN). The controls for the other concentrations have been omitted to avoid confusion, but in every case the volumes of the eggs in the controls were less than the volumes in the respective HCN solutions. In other words, the volumes of the eggs in the HCN solutions as shown in Fig. 1 are slightly greater than they would be if the HCN solutions had been entirely made up with sea-water. Since the KCN solutions were made up with sea-water only, these precautions were unnecessary for that series of experiments.

In order to test the narcotic properties of the cyanides, eggs were inseminated and after three minutes, placed in progressive dilutions of KCN and HCN. An *N*/100,000 HCN solution still inhibited cell division, while an *N*/30,000 KCN solution was the lowest concentration which would inhibit cell division. Eggs which had been inseminated and then narcotized, were replaced in sea-water and the time for first cell cleavage to appear noted. Eggs which had been exposed to various concentrations of both HCN and KCN for varying lengths of time, were then washed by letting them fall to the bottom of a test tube filled with sea-water and then transferred to sea-water in a watch-glass. These were then inseminated to see whether the eggs were still alive after the effects of the cyanides and the hypotonic sea-water.

The effect of KCN on the permeability was followed merely for comparison with the HCN and ten one-minute readings were taken of eggs in 50 per cent sea-water which had previously been exposed for 25 minutes to concentrations of KCN varying from *N*/300 to *N*/900. The KCN solutions were made up entirely with sea-water.

RESULTS.

Exposure of *Arbacia* eggs to HCN causes an increase in the volume of the eggs when placed in hypotonic sea-water, above that of the controls exposed to sea-water. The volume of the eggs varied directly as the concentration of the HCN and as the time of exposure to the HCN solutions. As previously stated, the fact that the HCN solutions were in themselves hypotonic in varying degrees, did not interfere greatly with the final results, since the increase in volume due to this hypotonicity was only slight as compared to the increase in volume due to the HCN. All eggs exposed to the HCN and to the sea-water controls finally reached the same equilibrium point, $4{,}518 \times 10^2\ \mu^3$. Only eggs which had approximately the same size at the beginning of the experiments, $2{,}381 \times 10^2\ \mu^3$, were used. The effect of the HCN was to hasten reaching the equilibrium point. Fig. 1, in which the volumes of the eggs after definite exposures to HCN are plotted against the time in the hypotonic sea-water, shows the rate at which the increase in volume occurs. Fig. 2 shows the

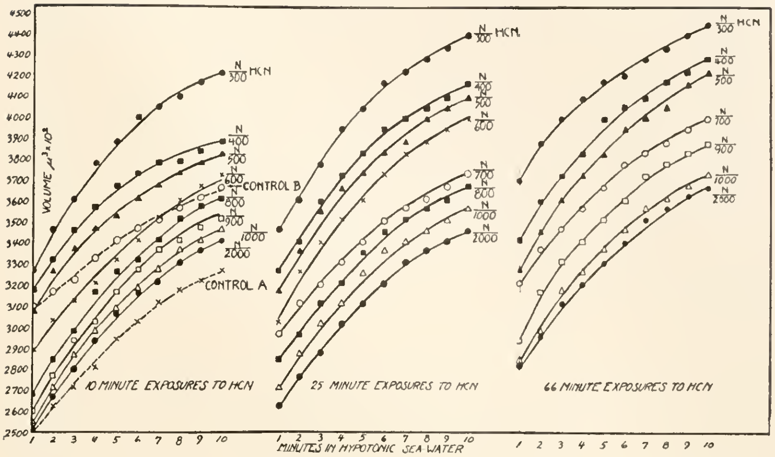


FIG. 1. Volume of eggs after definite exposures to various concentrations of HCN plotted against the time in 50 per cent. sea-water, showing the rate of increase in volume. Control A was exposed to 100 per cent. sea-water instead of HCN while control B was exposed to 67 per cent. sea-water, comparable to the hypotonicity of the $N/400$ HCN. Controls in other percentages of sea-water comparable to the remaining concentrations of HCN have been omitted to avoid confusion, but in all cases, the volumes of the eggs in sea-water were less than the volumes in the HCN solutions.

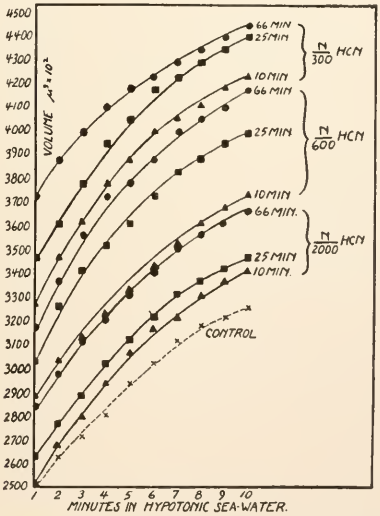


FIG. 2. Illustrating the rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying time intervals. The control was exposed to 100 per cent. sea-water.

rate of increase in volume of eggs exposed to the same concentrations of HCN but for varying lengths of time. Volume here is also plotted against time in hypotonic sea-water. KCN, on the other hand, caused a decrease in the volume of the eggs, when placed in hypotonic sea-water, below that of the control exposed to sea-water. The volume of the eggs varied inversely as the concentrations of KCN. Fig. 3 shows the rate at which the volumes of the eggs decrease with increase in concentration of KCN.

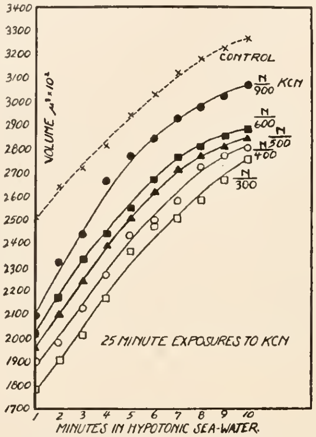


FIG. 3. Volumes of eggs after exposure to KCN showing the rate of decrease in volumes with increase in concentration of KCN. Control was exposed to 100 per cent. sea-water.

The pH of the HCN solutions varied only slightly, being 7.2 to 7.4, while the pH of the KCN solutions remained at 7.4. The work of Lucke and McCutcheon ('26*a*) shows that the volume of *Arbacia* eggs is independent of the pH of the solution except in cases where the pH is so high or so low as to injure or kill the eggs. According to the same authors ('26*b*) temperature does have an effect on the volume of the eggs in hypotonic sea-water. Although no attempt was made to keep the temperature constant during the experiments, an accurate record, taken every half hour during the experiments, was kept of the temperature of the laboratory. Conditions at Woods Hole are such that the temperature rarely varied ±2° from 22° C.

Eggs which had been exposed to *N*/300 HCN and *N*/300 KCN for 60 minutes, by which time they had reached their maximum or minimum volumes respectively, were returned to sea-water and fertilized. Cell division took place, showing that the eggs were alive at their greatest expansion or contraction. One cannot very well tell whether an anæsthetized egg can be fertilized while in that condition, since a normal egg does not show first cleavage until about 60 minutes after insemination. Within these 60 minutes, the narcotized condition may have been reversed, and the dividing egg be, not a narcotized egg, but a normal one. The anæsthetized egg, however, did form a fertilization membrane immediately after insemination, and in view of the statement which follows, might indicate that a narcotized egg can be fertilized, but cleavage is delayed until the narcotic has diffused out of the egg, or until the narcotized condition has been reversed. Untreated eggs normally showed first cleavage about 60 minutes after insemination. Eggs which had been anæsthetized with varying concentrations of HCN 3 minutes after insemination and then transferred to sea water, showed first cleavage at varying times always longer than the untreated egg; however, the higher the concentration of HCN used to anæsthetize them, the longer it took for first cell cleavage to appear.

Lillie ('16) has suggested a modification of the equation followed by unimolecular reactions $dx/dt = k(a - x)$, in dealing with rates of osmotic pressure in egg cell, of the form $kt = Ln\frac{V_{eq} - V_0}{V_{eq} - V_t}$ where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t. Lillie found that this equation represents the rate of swelling of fertilized and unfertilized *Arbacia* eggs in hypotonic sea-water and Lucke and McCutcheon ('26) found that it applied also to the rate of swelling in sea-water of varying hypotonicity.

That this same equation holds good in the series of experiments described in this paper, can be seen from Fig. 4. When log $\frac{V_{eq} - V_0}{V_{eq} - V_t}$ is plotted against time in hypotonic sea-water, a straight line should result. This has been found to be the case. The values of k, the velocity constant, are given by the slope of the line. This figure shows that the higher the concentration of

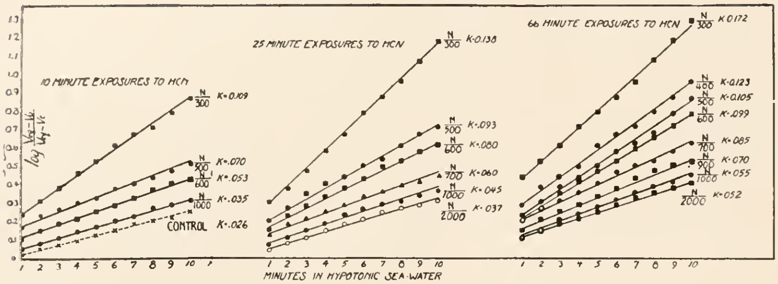


FIG. 4. Log $\frac{V_{eq} - V_0}{V_{eq} - V_t}$ (in which V_{eq} is volume at equilibrium, V_0 is volume at first instant, in sea-water, and V_t is volume at time t), plotted against the time in 50 per cent. sea-water. K, the velocity constant, is obtained from the slope of the lines.

HCN for any given length, the greater is the rate of swelling. Fig. 5 shows the rate at which the velocity constants increase with increase of concentration and increase in time of exposure to the HCN solutions. Fig. 6 is a composite curve in which all the velocity constants have been plotted to form a single curve. The various velocity constants have been plotted against their respective concentrations. The figure shows that the increase in the rates of reaction proceeds at a regular rate whatever the concentration of HCN and length of exposure.

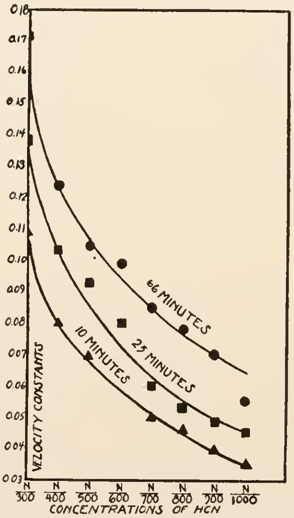


FIG. 5. Showing the rate of decrease of velocity constants with decrease in concentration of HCN.

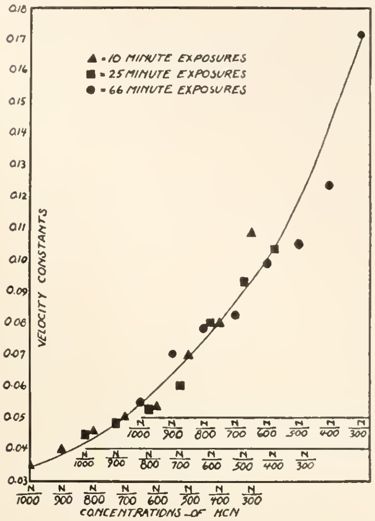
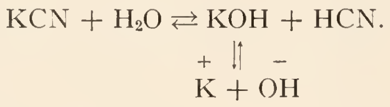


FIG. 6. All the velocity constants plotted against their respective concentrations to show that the velocity constants increase at a regular rate with increase in concentration of HCN and increase in time of exposure to these concentrations.

DISCUSSION.

Lillie's ('13) explanation of the phenomenon of antagonism by various anæsthetics and of anæsthesia in general is based on his experiments which showed that in every case the anæsthetics which he used prevented a general increase in permeability brought about by various salts which are toxic to the cell. That this decrease in permeability by anæsthetics does not always follow, at least when HCN is used as an anæsthetic, is shown by the preceding experiments. Heilbrunn ('25) has also found that ether in various concentrations increases the permeability of *Arbacia* eggs to water. The difference in the results obtained by Lillie and those reported in this paper may possibly be due to the difference in types of anæsthetics used. HCN, as is very well known, dissociates only very slightly. That it enters the cell as a molecule, as does CO_2 [Jacobs ('23)] and H_2S [Beerman ('24)], has been shown by Bodine ('24). Bodine suggests that HCN enters the cell as a molecule and ionizes within the cell to give an intracellular acidity even though the extra-cellular HCN solution is slightly alkaline.

It was shown that HCN is more potent as an anæsthetic than was KCN, since a greater concentration of KCN than HCN is needed to produce anæsthesia. One possible explanation of this follows: KCN in solution is alkaline due to the manner in which it dissociates:



$$\mathrm{KCN} + \mathrm{H_2O} \rightleftarrows \underset{\overset{+}{\mathrm{K}} + \overset{-}{\mathrm{OH}}}{\underset{\downarrow\uparrow}{\mathrm{KOH}}} + \mathrm{HCN}.$$

Thus it can be seen that a solution of KCN always contains a certain amount of HCN molecules. Therefore the anæsthetic property of KCN may be due to the HCN molecules present in it, but this HCN is partly antagonized, or its effects interfered with by the KOH formed at the same time. Why HCN should cause an increase and KCN a decrease in permeability is as yet problematic, unless it is the KOH in the KCN solution which is producing the decrease. It is hoped to gather more data on this point in the future.

That HCN acts primarily on the cell membrane and not on the interior of the cell, seems likely in view of the fact that eggs, whether treated with HCN or with sea-water, finally reach the same equilibrium point. From recent work Lucke and McCutcheon (personal communication) state that the velocity of swelling or shrinking of *Arbacia* eggs in hypotonic or hypertonic sea-water is the same, so that Heilbrunn's objection to calling an increase in the volume of the egg an increase in permeability on the ground that it may be an increase in the extensibility of the membrane, does not seem to hold. We would expect, from his statement, that the eggs would shrink faster than they would swell.

Summary and Conclusions.

(1) HCN in concentrations varying from *N*/300 to *N*/2,000 causes an increase in the volume of *Arbacia* eggs when placed in 50 per cent. sea-water, the rate of swelling varying directly as the concentration of HCN and the time of exposure to the HCN solutions.

(2) KCN in concentrations varying from *N*/300 to *N*/900

causes a decrease in the volume of the eggs when placed in 50 per cent. sea-water, the rate of decrease varying inversely as the concentration of KCN.

(3) Both HCN and KCN act as anæsthetics, the HCN being more powerful than the KCN.

(4) Anæsthetized eggs can be fertilized while in that condition but it appears that cell division is delayed until the anæsthetic has diffused out of the egg or until the narcotic condition has been reversed.

(5) The formula $kt = L_n \frac{V_{eq} - V_0}{V_{eq} - V_t}$ (where V_{eq} is volume at equilibrium; V_0 is volume at the first instant (in sea-water); and V_t is the volume at time t) correctly represents the rate of reaction.

(6) It is suggested that it is the HCN molecules present in a solution of KCN which causes anæsthesia by that salt, and that its lessened effectiveness is due to the antagonistic (?) action of the KOH which is present, at the same time, in an aqueous solution of KCN.

Appreciation is expressed to Dr. J. H. Bodine for suggesting the problem and for the helpful advice given the writer.

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THE LOW SEX RATIO IN NEGRO BIRTHS AND ITS PROBABLE EXPLANATION.

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It has sometimes been stated that the ratio of male to female births is lower among the Negroes than among whites. The records of births published by the U. S. Census Reports for 1880, 1890, and 1900, gave very low sex ratios for the Negroes,—namely 100.25, 102.67, and 99.80 respectively. The data on births for these years were admittedly incomplete. Births were estimated "by adding to the living childern under one year of age as shown by the population returns, the number of those who were born during the year ending May 31, but who died before the end of the year, as shown by the returns of deaths." The ridicuously low death rates estimated on the basis of the census returns for 1880 and 1890 made it evident that reports of deaths collected during the census year were very incomplete. The deficiencies in the census of 1880 were estimated by Dr. J. S. Billings as about thirty per cent., and those of 1890 were probably even greater. It is quite natural that failures to report deaths would affect most the data on the mortality of infants, especially among the Negroes. That this is the case is evinced by the very small proportion of infant deaths reported as compared with the number of living children under one year of age, whereas it is well known that the actual mortality rate among Negro infants is very high. Inasmuch as male infants suffer an exceptionally high death rate, the effect of basing estimates of births partly on data which leave out a large proportion of infant deaths is to give a sex ratio with too low a proportion of males. On account of the higher infant mortality of the Negroes, to say nothing of more numerous deficiencies in the records, the Negro sex ratio would be reduced more than that of the whites.

Aside from the rather unsatisfactory compilations published in the Census Reports we had, until a few years ago, relatively meager data on Negro births. Dr. J. D. Nichols has compiled records for the District of Columbia (1874–02) and finds a sex ratio in Negro births of 103.10. Beginning in 1915 the annual reports on birth statistics issued by the Bureau of the Census furnish a sufficiently large amount of data on the sex ratio of Negro births to give very reliable statistical results. The number of Negro births in the U. S. Registration Area from 1915 to 1923 inclusive totals 397,977 males and 386,348 females, giving a ratio of 103.01 males to 100 females. For the same period and area there were born among the whites 5,985,181 males and 5,651,287 females, giving a sex ratio of 105.91. The sex ratio of native-born whites was somewhat higher, 106.072, while that of foreign-born whites was slightly lower, 105.55. The sex ratio of children of mixed marriages, native and foreign-born, showed an intermediate figure,—105.84. The ratios for the Indians and the Japanese were 106.06 and 106.72, respectively, figures very close to the sex ratios of the Caucasians.[1]

Does the low sex ratio for Negro births indicate a peculiarity of race? The comprehensive data assembled by Gini point to the conclusion that the sex ratio constitutes a remarkably constant peculiarity of the human species. Before concluding, therefore, that the Negro sex ratio is essentially different from that of the white race other explanations should be sought for. Nichols has made the plausible suggestion that the low sex ratio among Negroes is a consequence of ante-natal mortality. If there is a greater ante-natal mortality among the Negroes than among the whites, and if this mortality is relatively higher in the male sex, there would naturally be a lower sex ratio among the live births in the Negro race. It is a well known fact that the sex ratio of still-births and abortions is unusually high. Data on still-births have been published by the Bureau of the Census for only three years, 1918, 1922, and 1923. These data are naturally very incomplete, and different states have various ways of defining and recording still-births. Nevertheless the data are quite illuminating in relation to the problem in question. The still-births and sex ratios for different groups of the population are shown in the following table:

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[1] The data on births for 1924, which have just come to hand, show much the same relations as those quoted. For total live births the ratio for Negroes is 103.98 and for all whites 105.95. The more recent data, therefore, bring the sex ratios for Negroes and whites more closely together. The sex ratios for still-births are as follows: total still-births, 137.48; total white, 137.96; native-born white, 137.81; foreign-born white, 145.26; native and foreign-born, 133.6; total colored, 135.17; Negro, 135.08.

STILL-BIRTHS IN THE U. S. REGISTRATION AREA IN RELATION TO RACE AND NATIONALITY.

		Total Still-births.	Total White.	Native White.	Foreign White.	Native and Foreign White.	Total Colored.	Negro	Indian.	Japanese.
1918	M.	28,127	24,837	15,489	6,413	2,148	3,290	3,257		
	F.	21,507	18,190	11,509	4,466	1,657	2,317	1,482		
1922	M.	39,672	33,593	21,150	8,254	3,190	6,079	5,925	53	91
	F.	29,173	24,739	15,785	5,829	2,374	4,434	4,332	30	59
1923	M.	39,566	33,511	21,232	7,986	3,302	6,055	5,925	37	76
	F.	29,235	24,770	15,840	5,831	2,436	4,465	4,360	32	64
Total	M.	107,365	92,301	57,871	22,653	8,640	15,424	15,107	90	167
	F.	79,915	67,699	43,134	16,126	6,467	11,216	10,174	64	123
Ratio		134.35	137.67	134.16	140.47	133.61	137.52	148.49	140.63	135.78

In all the groups, as may be readily seen, the sex ratio for still-births is remarkably high, and it is especially high for the Negro.

Now for the second point,—the relative proportion of still-births in Negroes and whites. Calculating the ratio of still-births to total births in the two races we find that the ratio is over twice as high among the Negroes as among the whites, namely 7.3 per cent. among the former and 3.5 per cent. among the latter. Syphilis, which is a potent cause of still-births and abortions, is very much more prevalent among the Negroes, and doubtless accounts in no small measure for the high Negro rate of ante-natal mortality.

The facts of differential race and sex mortality in uterine life enable us to explain the low sex ratio of live births among the Negroes without assuming that there is any real racial difference involved. In order to test this interpretation further I have added the still-births and live-births together in the two races and then calculated the sex ratios for total births. As would be expected, the differences between the sex ratios of the two races were reduced. For all whites combined the sex ratio became 106.72, and for the Negroes, 105.54. The inclusion even of the confessedly incomplete data on still-births wipes out most of the difference between the sex ratios of the two races. If complete data on still-births were available, the differences between the sex ratios would probably be reduced to insignificant proportions.

It is perhaps worth while to point out that the sex ratio of offspring resulting from the mating of native-born with foreign-born parents is lower that it is among the native-born. Such matings do not necessarily represent the union of distinct ethnic stocks to a much greater degree than the matings of either the native or the foreign-born, although they probably do so to a certain extent. If we may judge from my studies on the matings falling in this class among the parents of college students,[1] more than fifty per cent. of such mixed marriages would be between persons of the same extraction. On the whole, the federal statistics on births indicate that the sex ratio is little affected by the crossing of different ethnic stocks. There are several factors associated with educational, social, and economic status which probably influence the sex ratio to a greater degree.

The American Negro is to a considerable, but not precisely ascertainable, extent a product of the union of very distinct races. There are no extensive data on the sex ratio of mulatto births as compared with that of the more nearly pure blacks. But since mulattoes are relatively much more numerous in cities than in the country, one may compare the sex ratios of Negroes in urban and rural communities. I have done this for two years, 1922 and 1923, and have added the still-births and live births together. The sex ratio for the cities of the Registration Area is 103.78, and for the rural districts, 106.35. It would be unsafe to conclude, however, that race mixture lowers the sex ratio in this case. The relation is more readily explained by the higher proportion of still-births among the urban Negroes.

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Live Births and Sex Ratios at Birth in the U. S. Registration Area, 1915–22, According to Race and Nationality.

	Total Births.	Total White.	Native White.	Foreign White.	Native and Foreign White.	Total Colored	Negro.	Indian.	Japanese
M.	6,412,118	5,985,181	3,791,433	1,499,368	619,295	426,937	397,977	7,389	18,443
F.	6,064,740	5,651,287	3,574,396	1,420,482	585,110	413,453	386,348	6,967	17,281
Ratio.	105.73	105.91	106.07	105.55	105.84	103.26	103.01	106.06	106.72

THE CHROMOSOMES OF THE INDIAN RUNNER DUCK.

ORILLA STOTLER WERNER.

During recent years the attention of a number of cytologists has been focused on the problem of the avian sex-mechanism. Their investigations have brought varying results and numerous hypotheses have been advanced to explain the cytological findings in such a way as to bring them into agreement with the genetic evidence which is beyond dispute.

Cytologists agree that both physiologically and morphologically the character of the chromosomes of the birds is undetermined and that up to this time our only method of forming a conception of the activities of the chromatic material is through genetic study. In view of this consensus of opinion it would be a hazardous thing for a beginner in the field to criticize the theories that have been developed around the studies of the avian chromosomes unless he has a sufficient number of properly prepared figures for study and has subjected them to a very critical analysis.

Stevens, Hance, and Schiwago agree that the male chick appears to have two large chromosomes equal in size. These they presume are the X-chromosomes. Guyer claims to have evidence to show that in the guinea hen and in the domestic chicken the sperm are of two classes. These are to be distinguished by the presence or absence of an unpaired X-element or accessory chromosome. He believes, however, that the sperm which do not possess the X-element degenerate and are, therefore, non-functional. In regard to this Wilson (1925) says: "In view of this fact it is remarkable that the diploid group seems to show in the female but one large curved chromosome (X) while in the male two such elements are present. The diploid group thus seems to show the expected relations, the female being heterozygous, the male homozygous; but, per contra, the gamete formation of the male seems to show this sex to be cytologically digametic, with one class of gametes non-functional. It is stated, further, that the X-chromosome of the spermatocyte division is a bivalent body (representing the large pair in the spermatogonia) which passes as such to one pole. If these facts be correctly determined they offer a cytological puzzle with which it is not possible to deal without additional data."

For some time (June 1925 to June 1926) I have been engaged on a study of the chromosomes of the Indian runner duck and I offer the following data as a partial solution of the avian sex-chromosome problem.

Material and Methods.

The material used for giving mitotic figures consisted chiefly of the embryonic membranes of both sexes and to some extent, the germ cells of the male. In most cases the cells of the amnion afforded the best examples, although the cells of the chorion and the allantois gave good results; but such material is more difficult to prepare.

Tissues were obtained from individuals at different stages of incubation ranging from five to twenty-one days. However, those from eight to eleven days were found most suitable for the purpose. Before the eighth day stage it is difficult to determine sex and after the eleventh day the amnion has so completely formed that few dividing cells can be found.

Four general methods of technique were employed; whole mounts from embryonic membranes, stained sections of the embryos, sections of the testes, and smears of the testes.

Membrane Technique.—Of the embryonic membranes the amnion was found to give the best results. This is a very delicate tissue resembling a silk chiffon veil. Because of its delicate structure it was found best to keep it as nearly intact as possible. In this way the pressure of the amniotic fluid kept it stretched while being fixed and thus most of the tissue could be saved. Of the allantois only parts could be used. This was especially true of embryos of longer incubation, for the heavy blood vessels catch and hold the stain and the tissue is thick and becomes hard.

Great care was taken that the eggs should not become chilled while being removed from the incubator. The embryo within its amnion, and sometimes a part of the allantois, was removed from the egg with warm instruments and placed in Allen's modification of Bouin's fluid for two hours. The temperature of the fixative was kept at 37°. The amnion was punctured at the end of an hour to allow the fixative free access to the embryo.

The tissues of the first three embryos were rendered practically useless by increasing the strength of the alcohol too rapidly. The chromosomes were clumped and massed so that it was almost impossible to make a count. For this reason the following procedure was worked out. When the tissues were removed from the fixative they were rinsed in several changes of distilled water at a temperature of 37°, then passed successively through the following grades of alcohol: $\frac{1}{2}$, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 per cent. During this time the alcohols were maintained at the same temperature and the tissues were allowed to remain approximately twenty minutes in each fluid. While the material was in the 10 per cent. alcohol the membranes were removed from the embryos, and placed in a 2 per cent. solution of iron alum; one and one half hours. They were then rinsed in several changes of distilled water and placed in an aqueous solution of Heidenhain's hematoxylin, two hours; rinsed in tap water and destained in iron alum. They were then passed successively through the following grades of alcohol, remaining about ten minutes in each grade: 12, 14, 16, 18, 20, 22, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90 and 100 per cent. They were then passed through xylol, fifteen minutes, cedar oil three hours, back to xylol ten minutes, 100 per cent. alcohol one hour, forward to cedar oil two hours, then xylol fifteen minutes, and finally cut into small pieces for mounting. The double clearing makes the tissues beautifully transparent.

As suggested by Painter in his study of mammalian material, the large mesodermal cells were found to be the best for study. For this reason the tissue was placed on the slide with the mesodermal surface upward. They were mounted in gum damar, a small leaden weight being placed on the cover slip while the slides were drying.

Technique for Embryos.—The embryos were taken from the ten per cent. alcohol and passed through the same grades as were the membranes but because of their greater bulk were left thirty minutes in each grade. They were then passed through half

100 per cent. alcohol plus half xylol, 15 minutes; pure xylol, five minutes; one half xylol and one half 48° paraffin, fifteen minutes; then embedded in the usual way. They were cut at seven micra and stained with Heidenhain's hematoxylin and the work completed as is usual for such material. These slides were used in determining the sex of embryos whose membranes had furnished cells for study.

Technique for the Sectioned Material of the Testes.—The testes were from eight-months-old individuals. On removal from the body they were cut into millimeter cubes and dropped into the same fixative as above as soon as possible. They remained in the fixative two hours at 37° then were washed in several changes of distilled water at the same temperature. The material was now passed through the following grades of alcohol, two minutes each: ½, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, 20, 22, 25, 30, 35 and 40 per cent. At this stage all the alcohol was drained off except enough to cover the tissues. Then by means of a pipette, equal parts of bergamot oil and 50 per cent. alcohol were dropped in. The mixture was stirred constantly by bubbling it with a pipette. Again all the liquid was drained off except just enough to cover the tissues, and in each of the following changes this rule was observed. Equal parts of 60 per cent. alcohol and bergamot oil; equal parts of 70 per cent. alcohol and bergamot oil; pure bergamot oil. The old oil was now drained off and fresh bergamot oil dropped in. This process was repeated several times until all the alcohol was removed. The tissues were then left in the pure bergamot oil two hours (the tissues may be left in this oil for several hours without injury). The oil was then drained off and three fourths parts of bergamot oil and one fourth part of oil of wintergreen were dropped in, then one half bergamot and one half wintergreen, one fourth bergamot and three fourths wintergreen, pure wintergreen. Then the old wintergreen was drained off and fresh added several times until no bergamot remained.

From the oil of wintergreen the tissues were carried by steps through to paraffin. Sixteen bottles were used, starting with one part paraffin and fifteen parts wintergreen and finally ending in pure paraffin. Then the tissues were passed through several changes of pure paraffin to remove all the wintergreen. They were then placed in an oven for twelve hours, then in fresh pure paraffin for one half hour and finally embedded. While the tissues were being passed through the paraffin, the temperature was kept just so the paraffins would stay liquid, care being taken not to raise it above this point. Sections were then cut about five and one half micra. I think, however, that it would be better to cut them thicker than this, say about seven or eight micra. In this way many more cells could be found with none of the chromosomes sectioned away.

After the tissues were mounted the work was completed according to the usual methods.

The chromosomes in these cells stand out clear and beautiful.

The destaining is a delicate process and must be done with extreme care. No definite time can be given for the iron alum in destaining for the time depends upon the thickness of the sections, the concentration of stain used, etc. Experience alone brings the desired results. The work is best done under the low power of the microscope (10× ocular and 2/3 objective).

Technique for Testis Smears.—These were prepared just as the sections but the oils and the paraffins were omitted. From the 40 per cent. alcohol they were passed successively down through the grades of alcohol, care being taken to gradually lower the temperature of the fluids until when the work is completed they are at room temperature. This can best be accomplished by placing all the containers on an electric embedding plate.

The study of the avian chromosomes has presented difficulties in that the chromosomes are easily massed together, making a count difficult or impossible. With the technique here employed this difficulty has to a considerable degree been avoided.

Approximately six hundred cells were examined. The greater portion of these were soma cells from the tissues of nine individuals. In determining the number of chromosomes I have, in most cases, made drawings from several different slides. However, where the cells were found particularly clear and distinct I have made several drawings from the same slide.

The chromosomes were measured in the following manner. A separate camera lucida drawing was made of each. A thread was moistened and laid along the median curvature of the chromosome outline. By means of a razor blade its ends were cut at points corresponding to the boundaries of the ends of the chromosome as the thread lay upon the drawing. The segment was then removed and drawn out straight and measure taken in millimeters. In this way with a considerable degree of accuracy the length of the chromosomes was obtained.

Chromosomes of the Somatic Cells.

In the duck it will be more convenient to consider first the complex of the male (Figs. 1 to 7 and 43 to 48) since it is in this sex that the number of chromosomes is even.

There are probably seventy six. Some deviation from this number was found in the early part of the work. This was due, in greater part, to a failure to recognize the small globe-shaped chromosomes. For a time they were thought to be basophylic knots on the filaments that sometimes occur between the smaller chromosomes. As the work progressed, however, it became more apparent that these were true chromosomes. In addition to this deviation about half a dozen cells were found in the amnion in metaphase that appeared to have but half the usual number of chromosomes (thirty-eight). At this time no explanation can be offered for this condition. With the exception of the above few cases all cells examined, in which the chromosomes were well separated, seemed to have seventy-six. I am inclined to think that this is the basic number.

These seventy-six chromosomes fall readily into three general groups: six pairs of large chromosomes, including three **J**-shaped and three rod-shaped; nine pairs of short rod-shaped chromosomes; and twenty-three pairs of globe-shape. This may be seen in Figs. 43–48, Plate 8, which is an artificial pairing of these chromosomes arranged according to size. The morphology and the length were taken as a basis for this pairing. Where there is a slight difference in the lengths of the members of a pair, the shorter member is usually broader at one end than its mate. This makes the chromatic material of each member approximately the same.

Of the six pairs of large chromosomes (Nos. 38–33) the largest are J-shaped bodies (Figs. 3, 4, 6, 7 number 38). Next in size are a pair that are usually of rod-shape (Nos. 37) only slightly shorter than the longest in the complex. Pairs 36, 35, 34, and 33 form a graduated series in which there is but little difference in the lengths of the consecutive pairs (Figs. 1–7 and 43–48). However, there is a greater difference between pairs 34 and 33 than between the other pairs. Pairs 36 and 35 are of the **J**-type (Figs. 5, 6, and 7), and 33 and 34 are usually of rod-shape or bent rods, though in some prophase cells they seem to be **J**'s (Figs. 3, 7, 44 and 45).

The **J**-chromosomes (Nos. 38, 36, 35) of this group are, in most cases, constant in shape throughout prophase and metaphase and, as far as observed, in anaphase. The three pairs of rod-shaped chromosomes (37, 34, 33) assume various forms according to the stage of mitosis and according to their position in the nucleus. The members of the largest pair (Nos. 37) are especially interesting in this respect. In the metaphase they are, in most cases, straight rods pointed at the proximal end (Fig. 3). At times, however, they appear in the form of **U**'s (Fig. 4). If they lie near the nuclear wall they bend to accommodate themselves to this limitation. One or both ends may be bent (Fig. 7). Also these two bodies are not always bent in the same shape in the same cell but are so nearly the same in size that one may recognize them among the other chromosomes with a fair degree of certainty. Fig. 23 shows some of the shapes that they assume. There seems to be some differentiation of the chromatic material near the ends of these chromosomes which gives them increased flexibility at these points. This differentiation seems to be *a* suture accompanied by a slight constriction of the chromosomal wall. One of these sutures, if it is such, is near the incurved end when the chromosome lies as represented at *a*, *b*, *c*, *d*, in Fig. 23. The other point of differentiation is at the opposite end of the chromosome and marks off a portion of the chromosome about three times the length of the former end part.

With reference to the matter of sutures and constrictions, the condition found is not peculiar to the duck for such has been described by many observers in both animals and plants. These sutures and constrictions, it seems, may be median or at any other point and in some cases at least, they are constant in position. For example, Sakamura ('16) found that in *Vicia* several of the chromosomes show a constant subterminal constriction and that those of one pair show a constant median constriction in addition. However, Agar ('12) found in *Lepidosiren* that these sutures vary in position in different chromosomes and that they correspond with the points of attachment to the spindle and that their position in the chromosome of the meiotic division corresponds with that in the spermatogonial chromosomes. Wilson, however, points to the fact that these sutures and constrictions are visible in the prophase before the spindle is formed and therefore are not caused by the attachment. That these sutures may not be necessarily connected with the attachment of the traction fibers is shown by the No. 37 chromosomes of my material. Here there are two sutures, one at about one twelfth of the length of the chromosome distant from one end and the other at about one fourth the length of the chromosome distant from the other end. It is hardly probable that there would be two traction fibers so widely separated arising from the same somatic chromosome.

Again it should be pointed out that the permanence of position of such sutures at at least two different points along such chromosomes (see Fig. 23) would seem to be morphological evidence that such chromosomes differ in a qualitative way throughout their length.

So far, we have been following the characteristics, sizes, etc., of the largest chromosomes. Let us next consider the chracteristics of the chromosomes of the intermediate group. There are nine of these (Nos. 32 to 24) and they are of the short rod-shape type. Among these is one pair, the members of which are sometimes seen as crescent-shaped bodies (Fig. 8, cr.). Altogether the nine pairs form a closely graduated series ranging in size from those somewhat shorter than the shortest in the foregoing series to short ones that are almost as thick as they are long, but certainly distinguishable as rods by their acute angles (Figs. 43–48).

The third group of chromosomes consists of forty-six round or globe-shaped bodies (numbers 23–1). These also form a closely graduated series. Only twenty-two of these are of sufficient size to permit their being paired. The remaining twenty-four are small and so closely graduated in size that it is impossible to pair them with any degree of certainty.

The nine pairs of short rods and the twenty-three pairs of globe-shaped chromosomes are remarkably constant in shape during the progress of prophase from the time they are discernible as individual bodies until they enter the equatorial plate in metaphase. Here they mass together to such an extent that frequently they are no longer distinguishable as individual bodies. But even in this phase it frequently happens that they may be seen in their characteristic forms.

There appear to be seventy-seven chromosomes in the cells of the female (Figs. 8–22, 37–42). The longest of these is a large unpaired body, larger than any of the other chromosomes, and on account of its size, in most cases, easily distinguishable from the other chromosomes (Fig. 37–42, W). In cross section it is large at one end and taper at the other. In the earlier stages of mitosis it is a more or less bent rod, seeming to accommodate itself to the other nearby chromosomal regions and also to the nuclear wall (Figs. 8, 10, W). In the late prophase and the early metaphase it continues to be a more or less bent rod (Figs. 9, 11). In Fig. 17 it is a rod bent upon itself. Figs. 14 and 22 show it as **U**-shape while 16, 19 and 20 show it with bendings in different regions. In Fig. 12 it is a rod somewhat foreshortened. It would seem that this chromosome possesses a great degree of flexibility throughout its length which permits its bending in various ways.

The next largest chromosomes in the cells of the female take at times the form of curved rods but more frequently are of **J**-shape (Figs. 8–22, 37–42; 38w, 38Z). Whether they are gonial mates or not it is difficult to say. Theoretically, as will appear later, they should not be. From their size and their **J**-shape one would suppose that at least one of them is a homologue of the 38's in the cells of the male. The other might be of the nature of a w-chromosome.

Slightly shorter than these are two large curved rods (Nos. 37) one of which in prophase and early metaphase quite frequently takes the form of an **S** (Figs. 9, 17). The members of this pair resemble the pair number 37 in the cells of the male in sutures and constrictions (Fig. 23 a–d), and like them they assume various shapes (Fig. 23, e–i).

The remaining seventy-two chromosomes in the cells of the female (Nos. 36–1, Figs. 37–42) are approximately the same with respect to behavior, size relations, etc., as those in the cells of the male (Figs. 42–48).

In my study of the somatic cells I have been increasingly impressed with the evident pliancy of the chromosomes. They appear to bend to accommodate themselves to the nuclear wall and to other chromosomal regions. In most of the chromosomes (with certain exceptions to be mentioned later) the degree of pliancy seems to be about equal in all their parts. For this reason the morphology of chromosomes of the same length which are probably synaptic mates is not always the same. The ultimate conclusion regarding the shape of each particular chromosome, I believe, must be drawn from its appearance in metaphase where it is entering the equatorial plate and even in this phase some may be atypical in shape because of the obstructions of other chromosomes. The atypical forms, of course, have been found a lesser number of times and the conclusions are based on the larger number of cases.

The most convincing evidence is found in a comparative study of the chromosomes when those from different cells are arranged in serial order and in tables as in Plates 7, 8, and 9. Figs. 37 to 42 show the chromosomes from cells of the amnion of females; Figs. 43 to 48 from the cells of the amnion of males; Figs. 49 to 54, Plate 9, show first-spermatocyte chromosomes from smear preparations of adult testes. In these tables the constancy of the size and shape of the individual chromosomes stand out at once. There is some variation in form of the chromosomes from cell to cell but it is due, I believe, as previously stated, to their pliancy and in some instances to the particular angle at which the chromosome lies relative to the observer, and probably more than anything else to the particular stage of mitosis in which the cell happens to be found. Figs. 37, 38 and 40 are taken from early prophases (Figs. 8, 9, 10); Figs. 39, 41 and 42 are from prophases of later stage (Figs. 13, 12, 16); Fig. 43 is from a cell in early metaphase (Fig. 4); Fig. 47, and 48 are from late prophases (Figs. 1, 2).

The morphological groups and the size relations within the groups are very evident in this comparative series. The distinction in size, etc., between the group ot twelve (thirteen in the female) large chromosomes (38–33) and the group of 18 of intermediate size (32–24) is clearly marked in Plates 7 and 8. This is especially true in Figs. 37, 38, 40, 43, 44 and 45. It may also be plainly seen that the intermediate-sized and the globe-shaped chromosomes each form a finely graduated series. Because of this finely graduated condition and because there are so many of them there may have been some inaccuracy in the pairing and, in the case of the globe-shaped series, possibly in the count also, for these, the smallest chromosomes, frequently overlie one another. But it is certain that there is no inaccuracy in the count in the cases of the members of the larger and of the intermediate-sized groups. There is clearly 12 plus 18, or 30, of these bodies in the cells of the male and 13 plus 18, or 31, in the cells of the female. Neither can there be any doubt as to the presence of at least one unpaired chromosome among those from the female, for it is larger than and morphologically different from the other chromosomes. In the case of the chromosomes which have been arbitrarily numbered 38w and 38Z it might be said one is dealing with gonial mates, since they are similar in length and contain approximately the same amount of chromatin. These facts, however, considered alone, are not sufficient proof that they should be considered homologous rather than that they might be, as I shall attempt to prove later, two additional odd chromosomes which are concerned with the sex-mechanism. The important point in this paper is the fact, previously indicated, that a study of the cells of the male reveals an even number of the larger chromosomes while a study of the cells of the female shows an odd number and one more than in the cells of the male.

The morphological difference of the last group of chromosomes (globe-shaped) from the two preceding groups is not a condition peculiar to the duck. Painter shows the same condition in the lizard (Figs. 35, 36). As previously stated the twenty-two smallest chromosomes are so small and so closely graduated as to size that it is impossible to pair them. I have placed them in these plates simply to finish the series. In the first spermatocyte cells (Figs. 24–29) these globe-shaped chromosomes are picked out with less difficulty because they are fewer in number; but here the tetrad condition often interferes with distinguishing size relations. However, where a large number of such cells are studied a considerable degree of certainty concerning numbers, morphology, and size relations may be obtained.

Chromosomes of the Spermatocytes.

The preliminary process of reduction is initiated by syndesis in the course of which the chromosomes become closely associated two by two to form bivalents or gemini. This is, of course, only a pseudo-reduction producing a haploid group of bivalents each representing a pair of chromosomes. Because of this fact we would expect to find in the first spermatocytes where such phenomena occur one half the somatic number of chromosomes.

In counting, the prophase stages were found to be most suitable. Here the chromosomes lie well apart, which facilitates, not only the counting, but also a study of the forms of each, especially of the large tetrads (Figs. 24–29). Smear preparations were most suitable because whole cells could be used with the resulting certainty that all the chromosomes were present. Metaphase figures were also studied but no drawings of these are here used on account of the tendency of the small tetrads to crowd together in the center of the plate. The large chromosomes, however, stand out clearly in metaphase and have a tendency to occupy the outside of the circle as in the somatic cells (Figs. 1–22) and as in similar cells of other species (Figs. 35, 36).

Figure 24 represents a particularly clear prophase. In Fig. 49 the chromosomes of this cell are artificially arranged according to their size. In these bodies the tetrad form can be made out in most of the larger members, and at least the bivalent condition may be seen in the smaller members of the series. This is also true of Figs. 50 to 54. Attention is called to the similarity of the size relations among the members of the spermatocyte series to that of the homologous pairs in the somatic series (Plates 7, 8).

There is a distinct drop in size between the sixth and the seventh largest tetrads (Nos. 33 and 32). The same size distinction may be noted between numbers 33 and 32 of the soma cells. There is a similar distinction between the 35's and the 34's in the spermatocyte and somatic series. The size relations among the shorter spermatocyte rods (Nos. 32–24) and the bivalents derived from the globe-shaped chromosomes are apparently about the same as in the soma cells. In each case they form a similarly graduated series.

In addition to the resemblance in size between the spermatocytic and the somatic series there is also a striking similarity in form. Tetrad number 38, the largest in the group (Fig. 24), shows that it has arisen from a pair of **J**'s similar to those seen at 38Z in Fig. 43, Plate 8. In Fig. 25 the long arms of the **J** have not yet disjoined; the shorter arms have. This chromosome is well shown in Fig. 52 where the disjoining is almost complete. Chromosomes 36 and 35 also show that they have arisen from a pair of **J**'s (Fig. 25). In each of these chromosomes the long arms of the **J**'s have not disjoined while the shorter ones have. Chromosome number 37 (Figs. 24, 25, 26, 27, also Plate 9) shows constrictions similar to those found in number 37 in the soma cells, although these are more apparent in the tetrads, *i.e.*, the constrictions are deeper. In some of these cases only one of the constrictions is apparent but whether one or both are present, when they do appear, each seems to be in about the same position in the members making up the tetrad that it would occupy in the somatic homologues.

Gonomeric Grouping.

The probable existence of an odd chromosome in one of the gametes, as well as a definite number of the large chromosomes in each of the gametes, is further evinced in somatic cells in the grouping of the chromosomes in early metaphase. I have examined numerous cells in metaphase in both sexes. In the cells of the male the large chromosomes are grouped six on one side of the forming equatorial plate and six on the other (Figs. 3, 5, 6). In the cells of the female there are six on one side of the plate and seven on the other (Figs. 17, 18, 19, 20, 21, 22). In every case there is in the group of seven, one chromosome which is larger than the others which has the characteristic form of the largest odd chromosome in the cells of the female, large at one end and taper at the other.

Schiwago (1924) has recognized gonomeric grouping in the cells of the domestic chicken. (I have taken the liberty of reproducing some of his drawings as well as those of Hance from the same form and two from Painter from the lizard). The lettering of the chromosomes in Figs. 32 and 33 are as Schiwago gave it. The same grouping is apparent in the work of Hance and Painter although the authors do not point out the fact. I have again taken the liberty to draw a line through each of these figures (Figs. 30, 31, 35, 36) which might suggest a possible separation of the chromosomes into maternal and paternal groups.

D. H. Tennent (1908) found gonomeric grouping when working on the eggs of *Toxopneustes* and *Arbacia* fertilized with *Moina* sperm. Margart Morris (1914) in her work on hybrids between *Fundulus* and *Ctenolabrus* found a somewhat similar condition. Many other instances of this phenomenon among different species are on record.

Gonomery represents no more than a tendency on the part of the chromosomes to remain in separate maternally and paternally derived groups during a part of the early development. Considering these facts, such a phenomenon in the duck would seem to afford a valuable check on the count of the chromosomes, as well as a check on the number contributed by each sex, especially with respect to the large chromosomes, since these are so well defined that they may be readily recognized.

Filamentous Linkage.

Filaments which seemed basophylic in character were found connecting chromosomes in somatic cells in prophase stages. Not all groups of chromosomes formed by such fastenings were constant. Some groups composed of definite numbers were found a sufficient number of times to justify one in concluding that they might be relatively constant. In nearly all cases the groups are made up of chromosomes of the intermediate and smaller sizes. In some cases chromosomes especially of the short rod-type might be connected by two such filaments in such a way as to extend one from either corner of the end of an individual chromosome and attaching in like manner to the end of the next chromosome. In other cases both filaments might be fastened at the same point on a rod-shaped or a globe-shaped chromosome (Fig. 10).

Of the inconstant groups one composed of eighteen small chromosomes may be seen in Fig. 9. In the upper part of this figure there is a group of two chromosomes attached to one of the large J-shaped chromosomes. Fig. 13 shows a group of 13. Sometimes filamentous fastenings occur between the smaller chromosomes when these bodies lie in what is, apparently, a linear arrangement. However no cells have been observed in which all the chromosomes so arranged were fastened by filaments. If such a condition exists, it would be exceedingly difficult to discover since the chromosomes so arranged lie close together and are usually at two foci, the one lying over the other or nearly so.

Of the groups having the more constant types of fastenings one may be seen in Figs. 1, 9, 10 and 12. In this group ten of the chromosomes belonging to the intermediate series, seem to be connected end to end by interchromosomal filaments in such a way as to form two chains of five chromosomes each, consisting each of two rods and three globes. The members of the one series are similar in size to those of the other group. There are also in late prophase, four chromosomes belonging to the intermediate series connected end to end in such a way as to form two chains of two chromosomes each (Figs. 1, 9, 12).

Quite often the greater number of the small chromosomes appear to be in linear arrangement, I believe that in some cases they extend in a circle within the nucleus. In late prophase there seems to be a row extending almost across the space within the circle of the large chromosomes (Figs. 8, 9, 12). In some cases this row bends a little as a line appears when it extends around a globe. Manipulation of the fine adjustment shows that another row similar to this one lies at a deeper focus (Fig. 12) apparently at the opposite side of the nucleus. (It must be borne in mind that I was examining entire cells, not sections of cells.) These rows may extend in any direction across the nucleus, but they always lie within the circle of the larger chromosomes.

Discussion.

The chromosome number in the somatic cells and early germ cells is traceable half of it to the paternal parent and half of it to the maternal parent. There is, then, in the typical cell, an even number of chromosomes. If in any form an odd number is found, the question is immediately presented as to the disposition of the odd chromosome in maturation and what part it might play in the transmission of hereditary factors.

An attempt to solve the problem of the odd chromosome in the case of the Indian runner duck, as well as in any other form, necessitates not only a tracing of its disposition or probable disposition in maturation and zygote formation but also a discovery of a parallelism with the vast amount of results obtained from experimental breeding.

In the Indian runner duck the male has an even number, seventy-six chromosomes, but the female has an odd number, seventy-seven, and strange to say, one more rather than one less than is present in the male. Of course where the odd number occurs in the female, one would immediately look to the chromosomes in polar body divisions for an explanation. The difficulty of getting eggs in the right stage of maturation is at once apparent. It seems best, therefore, at this time to attempt an explanation of the phenomenon from indirect evidence and in the light of the results of the work on Lepidoptera, in which the female is known to be digametic for sex as is probably the case in the aves.

In the duck the additional unpaired chromosome present in the cells of the female is without a homologue in the cells of the male and of course could not then be a sex-linkage chromosome. If it is not concerned with sex-linkage it must probably belong to the class of sex-determining bodies known as the W-chromosomes, occurring only in females.

With this chromosome (W) ruled out of consideration it then becomes the problem to find which one of the remaining chromosomes in the female is concerned with sex-linkage and finally to account for the presence of a third non-homologous body which must evidently be present.

Sex-chromosomes carrying sex-linked characters are usually among the largest of the complex. In the male of the Indian runner duck, then, it is reasonable to suppose that the sex pair would be among the largest if not the largest in the series. In this series there are certainly six pairs that are sharply marked off in size from the remaining thirty-one pairs. Among them again three pairs may be distinguished as larger than the remaining three, and the largest of these is a pair of **J**'s which it might be supposed are the sex pair. For the sake of argument, at least, let us assume that 38Z is the sex pair (Figs. 37–42, number 38Z).

Now, in the female the sex-chromosome concerned with sex-linkage should be an unpaired body and similar in size and shape to the sex pair in the male with the members of which it, of course, would have to alternate. Evidently the largest chromosome (W) can not be this body. If the sex-linked chromosomes in the male are the largest in the series, as is usual in animals, in the cells of the female we would of necessity look for a homolgue of them among the largest in the series after the longest chromosome (W) is ruled out. From the drawings it would seem that there are four of these next largest chromosomes and they are very much alike in size. Just what one of the four might function in the capacity of a sex-linkage body is not possible to determine now. It might be supposed, however, that, as in the male, it is one of the largest (38Z).

If that be the case there would then remain an unpaired chromosome in the female to be taken from the remaining three large chromosomes. This chromosome like the largest one (W) in the female series would have no homologue in the male series and of course would be limited to the female line and would be a second w-chromosome (w).

In the female complex, then, there would be a group of three large chromosomes, one of which would be a sex-linked chromosome, homologous to and capable of alternating with the largest sex-linked chromosomes in the male. The other two would be chromosomes not concerned with sex-linkage and, since they remain in the female determining gametes, would be concerned only with sex-determination.

If the large unpaired chromosome in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the condition in the duck might then be a case similar to that in *Phragmatobia*, one of the Lepidoptera, which was described by Seiler (1914).

In *Phragmatobia* during polar body formation a single element was seen to separate from a large heteromorphic element that consisted of two unequal chromosomes yoked together. After separation the heteromorphic element fragmented into two unequal chromosomes at one pole. As the result, half of the eggs got the large single element and half got the two unequal chromosomes.

If the duck be like *Phragmatobia*, the unpaired chromosome here designated as Z might be thought comparable to the large element in *Phragmatobia* and some two of the other large chromosomes, here designated W, w, might be comparable to the heteromorphic element in *Phragmatobia* which was seen to be distributed to one pole in maturation and to break into two chromosomes.

Sex-Determination and Sex-Linkage.

Wilson (1925) is of the opinion that "all the difficulties" in regard to an explanation of the function of sex-chromosomes in sex-determination" disappear is we assume that in any particular species there is but one kind of X-chromosome, in itself neither male-determining nor female-determining, but so adjusted to the general mechanism of development that when single it swings development toward the male side, when double toward the female side. This view essentially quantitative ascribes to the egg the capacity to produce either the female or the male, according to the presence of more or less of the X substance."

On the other hand, Bridges (1922) from his study of triploids in *Drosophila* concludes that "both sexes are due to the simultaneous action of two opposed sets of genes, one set tending to produce the characters we call female and the other to produce the characters called male." According to his hypothesis there is a preponderance of male tendency genes located in the autosomes and a preponderance of female tendency genes located in the X-chromosomes. In the presence of one X the male-tendency genes located in the autosomes overbalance the female tendency genes in the single X-chromosome and a male results; on the contrary the presence of two X-chromosomes overbalances the male-tendency genes in the autosomes and the development is thrown toward femaleness.

Goldschmidt (1923) in his work on moth hybrids has come to the conclusion that the female producing factors lie in the W-chromosome. In crosses between certain races females are gotten through non-disjunction in such a way that the W-chromosome comes from the father and the Z-chromosome comes from the mother, just the reverse of the usual method. In such cases he finds that femaleness follows the W-chromosome.

In the light of the results obtained by Bridges on sex-intergrades in Drosophila produced through triploidy or otherwise unbalanced conditions of chromosomes, it is very clear that sex is not controlled entirely by a particular chromosome either singly or doubly represented as Wilson would say. There may be genes in many of the autosomes as well as in the sex-chromosomes which individually tend to throw the balance toward maleness or toward femaleness. But Bridges was dealing with an X–Y type of sex-determination and with male digamety. His opposing tendencies are located in the X-chromosomes and the autosomes. In addition to this his X-chromosomes carry sex-linked characters. Bridge's scheme as may be seen, will not work for the birds or moths where female digamety occurs But if we adopt the suggestion of Goldschmidt (1923) that the W-chromosome which passes from mother to daughter carries. female-tendency genes only and in addition assume that male-tendency genes are located in the Z-chromosomes of which two occur in the male and one in the female, we have a mechanism that in many cases would seem to take care of both sex-linked characters and sex-determination.

In the duck then, if the large unpaired element in the cells of the female is one of a complex of three that go to make up the sex-determining mechanism, the following scheme would be entirely in accord with the parallelism of cytology and experimental breeding.

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In the male there are seventy-six chromosomes. It would seem then that seventy-four of these are autosomes and two are Z-chromosomes. In maturation each sperm would get thirty-seven autosomes, plus one Z-chromosome, or thirty-eight in all, and all male gametes would therefore be alike.

In the female there are seventy-seven chromosomes. Comparing these with the conditions in the male it would seem that there are seventy-four autosomes, plus one Z, like those in the male, plus two W-chromosomes which are not present in the male.

In maturation it might be supposed that the two W's become linked together and that one of these might pair with the Z-chromosome, the whole resulting in a tripartite body. If the thirty-seven pairs of autosomes behave as in the male there would result gametes in the female of two sorts (Figs. 55, 56) one having thirty-seven autosomes plus the Z and the other having thirty-seven autosomes plus the Ww pair. At the formation of the zygotes then, those eggs having thirty-seven autosomes plus the Z, fertilized by a sperm having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus two Z's or a male. Those eggs having thirty-seven autosomes and two W's fertilized by a sperm as before having thirty-seven autosomes plus a Z would result in a zygote having seventy-four autosomes plus one Z and two W's or seventy-seven chromosomes and would be a female.

In sex-linkage the Z-chromosomes alone would be concerned. If the double lined Z, represented in Fig. 55 as occurring in the male, is thought of as carrying a dominant sex-linked character and the single lined Z here represented as occurring in the female, be thought of as carrying a recessive sex-linked character, then in the F_1 generation, both males and females would show the dominant sex-linked characters and would be like the male of the parental generation. But in the F_1 generation all males would be heterozygous for the sex-linked characters and would form gametes of two sorts, half carrying the dominant sex-linked character and half the recessive.

In the F_2 generation all males would show the dominant sex-linked character, but of the females of this generation, half would show the dominant sex-linked character and half would show the recessive character, the female inheriting it in each case from the male parent of the F_1 generation.

According to this scheme it might be supposed that in the case of the Indian runner duck each autosome sustains a balanced condition of the male and female-tendency genes. The Z-chromosomes might be thought of as carrying a preponderance of male-tendency genes. An individual, then, receiving two Z's would become a male. The two W's might be thought of as carrying female tendency genes only. These, however, would be thought of as being quantitatively greater than the male tendency genes carried in a single Z and an individual receiving a Ww plus one Z would become a female.

A large number of animals belong to the X–Y type in sex-determination. In these the female has an even number of chromosomes, the X-chromosome being paired or diploid. The male has one less than the even number of chromosomes found in the female, the X being unpaired. This gives the female more chromatin than the male. In a few of the animals of the WZ mechanism, such as some of the moths, the male has an even number of chromosomes. The female has the odd number and one less than the male which would give the male more chromatin than the female. But in most of the cases in the moths the female has one more chromosome than the male, the male having the even number.

In the birds, if my observations are borne out on other species, it would seem that the female has the preponderance of chromatin. If the conditions in the duck are representative of that which one might expect to find generally in the aves, we would expect in the moths which are likewise digametic in the female, that the true state of affairs would be like those moths in which the female has one more chromosome than the male. The difference between the two types of animals, the XX–XY and the WZ–ZZ, is essentially in the matter of which sex produces the two sorts of gametes. And so far as this mechanism is concerned it is a matter of indifference which sex produces the two sorts of gametes. The important thing seems to be that one sex, usually the female, should have more chromatin than the other.

SUMMARY.

1. This study was undertaken with two points in view: First, to determine the number of chromosomes and second, to discover the mechanism for sex-determination and sex-linkage in the duck as a representative of the aves.

2. Material from thirteen individuals was used, seven males and six females.

3. Chromosome counts were made on entire cells, both somatic and spermatocytic.

4. In the somatic cells there appear to be seventy-six chromosomes for the male and seventy-seven chromosomes for the female. There is present in the cells of the female a long unpaired chromosome which is not found in the cells of the male. There is reason to suppose that there are probably among the remaining six largest chromosomes two more unpaired chromosomes, one of which, the largest, is probably homologous to the largest pair (sex-linkage) of chromosomes in the male complex, while the other, it is thought may be some one of the five remaining long chromosomes.

5. There appear to be 38 bivalents in the primary spermatocytes of the male. These agree with the pairs of somatic chromosomes in size gradations. Most of the chromosomes in the spermatocytes appear to be bivalent or tetrad in form.

6. Sutures accompanied by constrictions seem constant in position in at least two points in the largest rod-shaped chromosomes. These sutures seem to lend additional pliancy to these regions in the chromosomes.

7. Gonomeric grouping of chromosomes occurs in the amnion cells of the duck. It is also thought to be present in the chromosome plates of the gonial cells in the embryo of the chick and the lizard.

8. Filamentous linkage between certain of the smaller chromosomes appears to be present in certain stages of the prophase.

9. There is reason to believe that the sex-mechanism is of the WwZ–ZZ type similar to that found in the moth Phragmatobia.

10. This would give the female more chromatin than the male and yet preserve female digamety, which would bring this type into harmony with the usual conditions found in the XX–Xy type.

Acknowledgment is hereby made to Dr. W. R. B. Robertson, at whose suggestion this problem was undertaken, for advice and assistance during the progress of the work and for a sympathetic understanding of the difficulties of the task undertaken.

University of Missouri,
Columbia,
September 1, 1926.

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EXPLANATION OF PLATES.

All figures from the Duck are reproduced at the same scale. The drawings were outlined with an Abbe camera lucida at a magnification of 3,500 diameters, obtained with a Spencer 1/12 homogeneous immersion objective and a Zeiss 18x compensating ocular with draw tube set at 150 mm. and drawing made at the level of the base of the microscope. The drawings were then enlarged by means of a copying camera lucida to 7,350 diameters. Having been reduced one half in the reproduction, they now appear at a magnification of 3,675 diameters.

W, the large sex-chromosome carrying female-tendency genes only.
w, the smaller sex-chromosome which carries likewise female-tendency genes only.
Z, sex-chromosome carrying a preponderance of male-tendency genes and also sex-linked genes.
cr, cresent-shaped autosomes No. 32.
38Z, same as Z.
38w, same as w.
37 to 1, autosomes.

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Plate I.

Figs. 1 to 6. Cells from the amnion of males of the Indian runner duck. The Z or sex-chromosome is numbered 38Z in each of the cells. 76 chromosomes present.

Fig. 1. Late prophase. The large chromosomes are shown in the characteristic peripheral position with the smaller ones within the circle.

Fig. 2. An earlier prophase than 1.

Figs. 3, 5, 6. Cells in metaphase. The stippled line in each case indicates a possible grouping of the chromosomes in maternal and paternal groups. This grouping in each case has been considered with especial reference to the 12 largest chromosomes. The autosomes paired from 37 to 33, according to size.

Fig. 4. An early metaphase. Autosomes paired from 37 to 23.

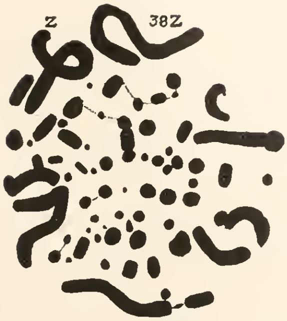


FIG. 1

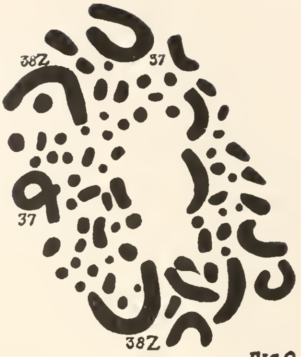

38Z
37
37
38Z

FIG. 2

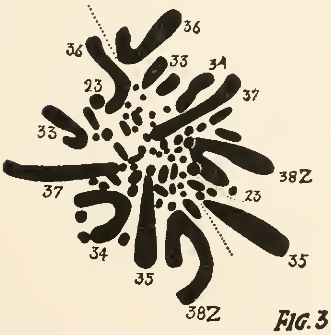
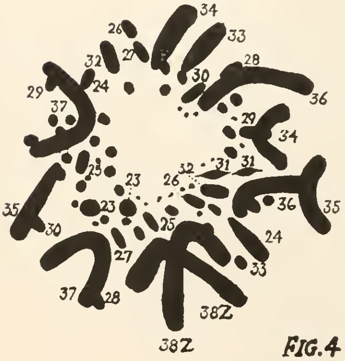

36
36
33
34
23
37
33
38Z
37
23
34
35
35
38Z

FIG. 3


34
26
33
32
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37
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31
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27
33
37
28
38Z
38Z

FIG. 4

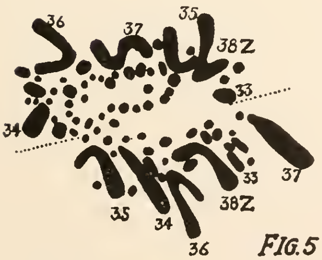
36
37
35
38Z
33
34
33
37
38Z
35
34
36

FIG. 5

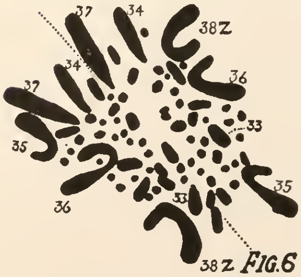
37
34
38Z
34
36
37
33
35
35
33
36
38Z

FIG. 6

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PLATE 2.

FIG. 7. Metaphase of a cell from the amnion of a male Indian runner duck. The sex-chromosome is numbered 38Z. Autosomes from 37 to 33 are paired according to their size.

FIGS. 8 to 12. Cells from amnion of females of Indian runner duck. W and 38w are the chromosomes that are supposed to carry female-tendency factors only. Some filamentous linkage among the smaller chromosomes is shown in each cell. Autosomal pairs, numbered from 37 down, indicate their relative size in decreasing series. 77 chromosomes present.

FIG. 8. Prophase. Autosomes numbered from 37 to 33. Autosomes 29 are here designated *cr* to indicate their crescent form.

FIG. 9. Late prophase. Autosomal pairs from 37 to 1 indicated. The two groups of five small chromosomes each are shown at the upper left side of the cell.

FIG. 10. Prophase. Autosomes from 37 to 33 numbered.

FIG. 11. Prophase. The place of sutures in No. 37 is well indicated by bendings of the chromosomes in this cell. Autosomes from 37 to 33 numbered.

FIG. 12. Prophase. W in this cell is somewhat foreshortened.

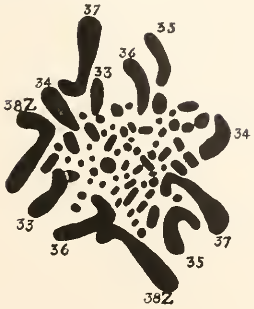
37
35
36
33
34
38Z
34
33
36
37
35
38Z

FIG. 7

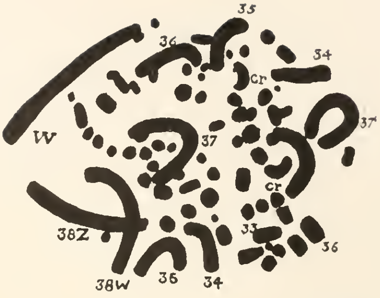
35
36
34
cr
37
W
37
cr
33
36
38Z
38W
35
34

FIG. 8

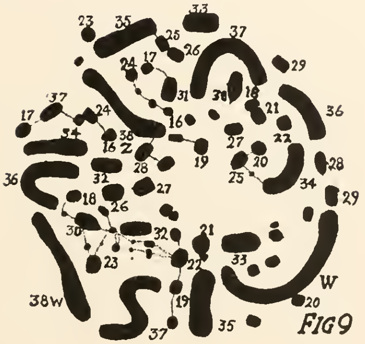
23
35
33
37
25
26
29
24
17
31
38
18
21
36
37
17
24
16
34
38
Z
16
27
22
28
19
20
28
36
32
25
34
29
18
26
27
30
32
21
23
22
33
W
38W
19
20
37
35

FIG 9

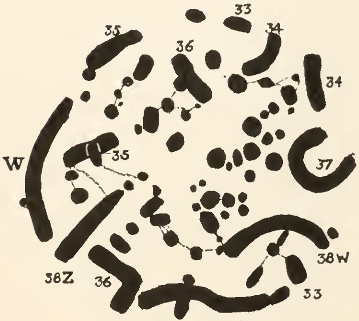
33
35
34
36
34
W
35
37
38W
38Z
36
33
37

FIG 10

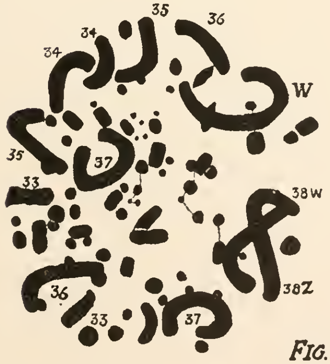
35
36
34
34
W
35
37
33
38W
36
38Z
33
37

FIG. 11

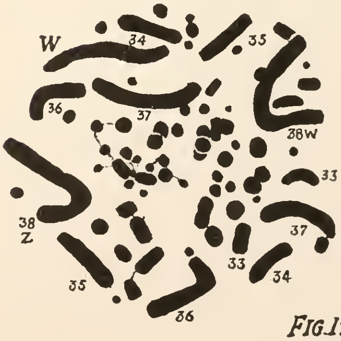
W
34
35
36
37
38W
33
38
Z
37
33
34
35
36

FIG 12

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PLATE 3.

FIGS. 13 to 18. Cells from the amnion of females.

FIG. 13. Prophase. Autosomes from 37 to 33 numbered.

FIG. 14. Prophase. This cell is atypical in the arrangement of the large chromosomes. However, 77 chromosomes are present.

FIG. 15. Prophase. Autosomes 37 to 33 paired.

FIG. 16. Prophase. Autosomes from 37 to 1.

FIG. 17. An early metaphase. The W chromosome is bent upon itself.

FIG. 18. A late metaphase showing especially gonomeric grouping. The most of the large chromosomes in this cell are foreshortened.

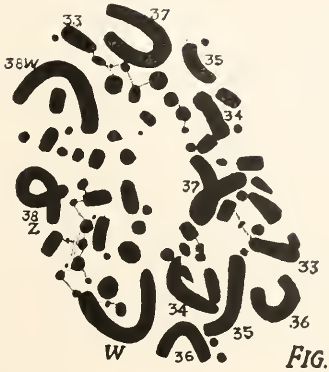
33
37
38W
35
34
37
38
Z
33
34
36
35
W
36

FIG. 13

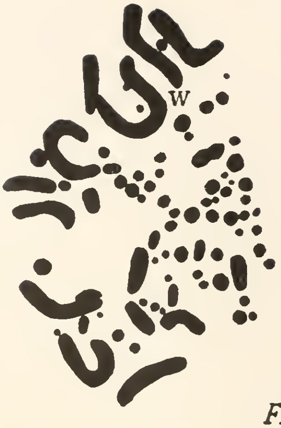

FIG. 14

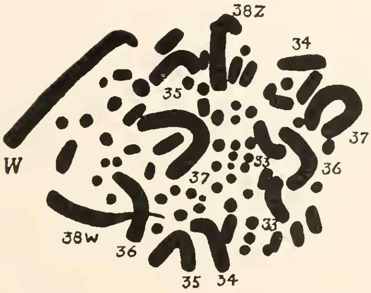
38Z
34
35
W
37
33
36
37
38W
36
33
35
34

FIG. 15

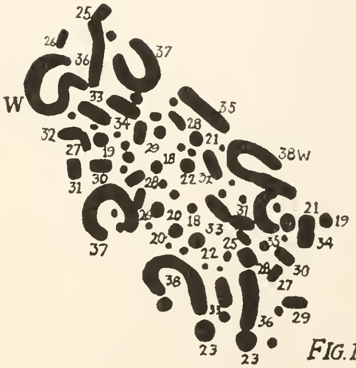
25
26
36
37
W
33
35
34
28
32
27
19
29
21
38W
18
30
22
31
31
28
26
20
18
31
21
19
20
33
25
35
34
22
28
30
37
38
27
31
36
29
23
23

FIG. 16

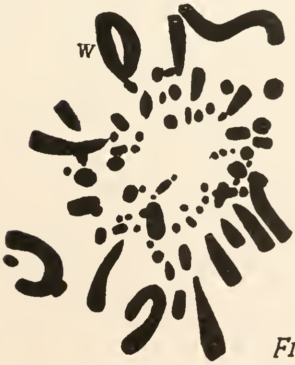

FIG. 17

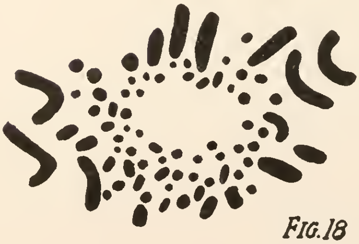

FIG. 18

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PLATE 4.

FIGS. 19 to 22. Cells from the amnion of females showing gonomeric grouping of the chromosomes.

FIG. 23. This figure shows some of the forms assumed by chromosome number 37; also the location of the sutures. Figures a, b, c, d are from cells of the male, and figures e, f, g, h, i are from cells of the female.

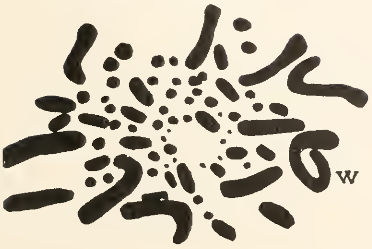

FIG. 19

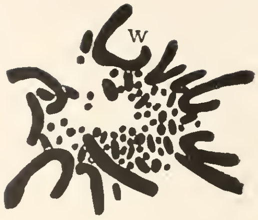

FIG. 20

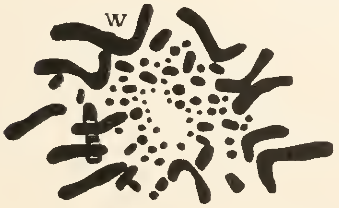

FIG. 21

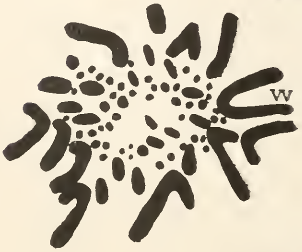

FIG. 22

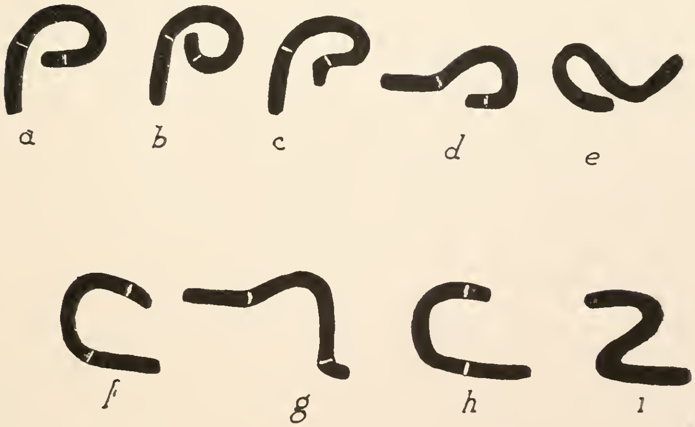

FIG. 23

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PLATE 5.

FIGS. 24 to 29. First spermatocytes from smear preparations. The Z-chromosome bivalent in each cell is numbered 38Z, the autosomal bivalents from 37 to 1 according to their size. The cells are in the prophase stage.

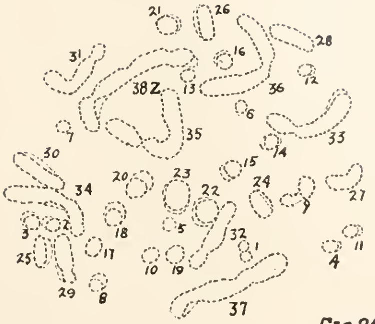

21
26
28
31
16
38Z
13
36
12
6
7
35
33
14
30
15
20
23
24
34
22
27
9
3
2
18
32
1
11
25
17
10
19
4
29
8
37
5

FIG. 24

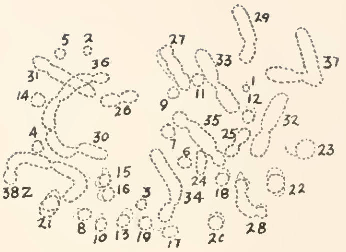

29
5
2
27
33
37
36
31
14
11
1
26
9
12
35
32
25
4
30
7
23
6
15
38Z
16
3
24
18
22
34
21
8
10
13
19
17
20
28

FIG. 25

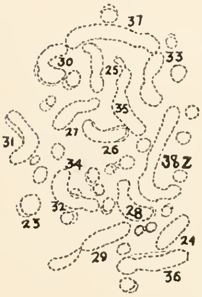
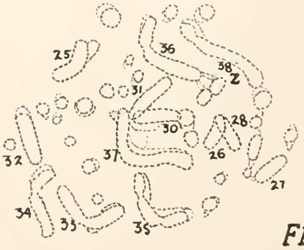

37
30
33
25
35
31
27
26
34
38Z
32
23
28
24
29
36

FIG. 26


25
36
38
Z
31
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28
26
32
37
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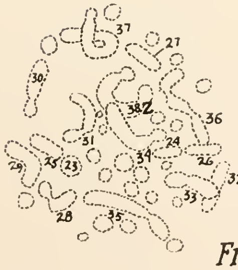
34
33
35

FIG. 27


37
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38Z
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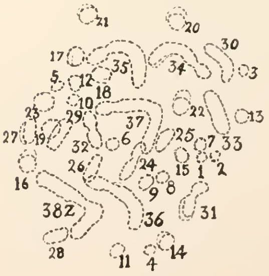
FIG. 28


21
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34
3
5
12
18
10
22
13
23
29
37
27
19
32
6
25
7
33
26
24
15
1
2
16
9
8
38Z
36
31
28
11
4
14

FIG. 29

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PLATE 6.

FIG. 30. Chromosome complex taken from embryonic female gonad of chick. After Hance. In the original drawing the large chromosome at the top of the cell is labeled A to indicate that it is the largest in the complex. It is not paired in the cells of the female. I have added the line to the drawing to indicate a possible gonomeric grouping.

FIG. 31. Chromosome complex taken from a cell in an embryonic male gonad of the chick. After Hance. In the original drawing the two largest chromosomes at the top of the cell are labeled A, a, to indicate that they are the largest in the cell. They are thus present in duplicate in the male. I have added the line to indicate a possible gonomeric grouping.

FIG. 32. Somatic cell from the female of domestic chicken showing similar grouping. After Schiwago. The lettering is according to Schiwago and is his indication of the "paternal" (right) and "maternal" (left) grouping of the chromosomes.

FIG. 33. Somatic cell from the male of domestic chicken showing same. After Schiwago. The indications are as in figure 31.

FIG. 34. Spermatogonia cell of domestic chicken. After Stevens. The 12 large chromosomes are arranged in peripheral position. I have drawn no line here to indicate gonomeric grouping but such grouping is obvious.

FIGS. 35 to 36. Somatic cell from embryo of lizard (*Sceloporus spinosus*). After Painter. I have again added lines to each of the drawings to indicate possible gonomeric grouping.

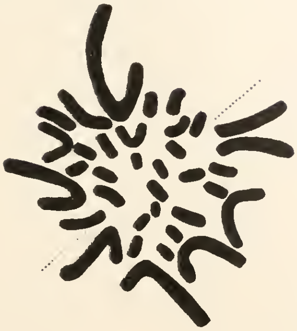

FIG. 30

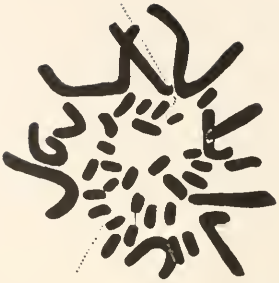

FIG. 31

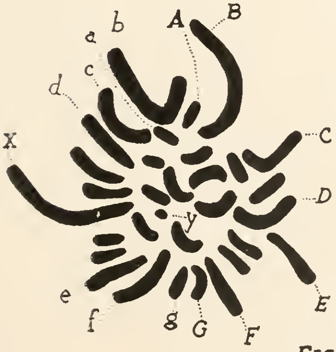
a
b
A
B
c
d
X
C
D
y
e
f
g
G
F
E

FIG. 32

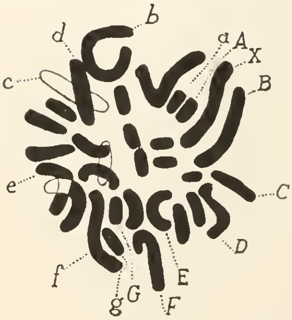
d
b
a
A
X
B
c
e
C
D
f
g
G
E
F

FIG. 33

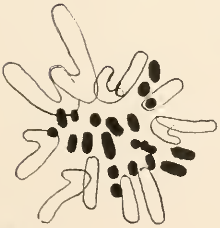

FIG. 34

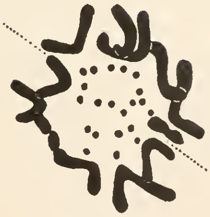

FIG. 35

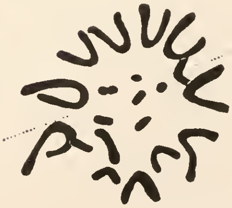

FIG. 36

ORILLA STOTLER WERNER.

Plate 7.

Figs. 37 to 42. Chromosomes from the amnion of females artificially arranged according to size. W, w indicates chromosomes carrying female-tendency genes only. 38z indicates the chromosomes carrying sex-linked factors and supposedly a preponderance of male-tendency genes.

Fig. 37. Chromosomes from a prophase shown at Fig. 8. Autosomes 32 are here crescent-shaped.

Fig. 38. Chromosomes from a late prophase shown at Fig. 9.

Fig. 39. Chromosomes from a prophase shown at Fig. 13.

Fig. 40. Chromosomes from a prophase shown at Fig. 11.

Fig. 41. Chromosomes from a prophase shown at Fig. 12.

Fig. 42. Chromosomes from a prophase shown at Fig. 16.

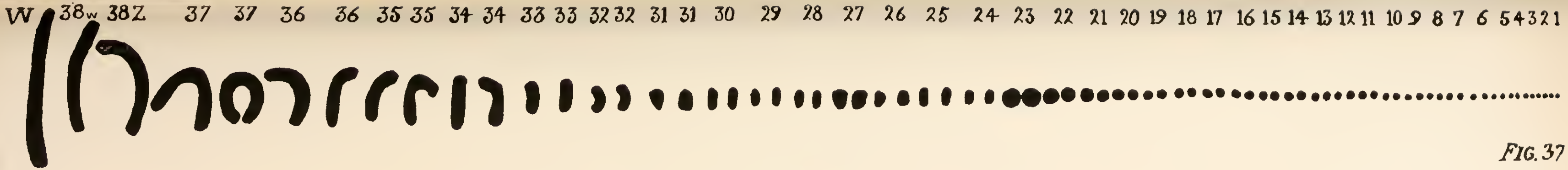
W 38w 38Z 37 37 36 36 35 35 34 34 33 33 32 32 31 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 37

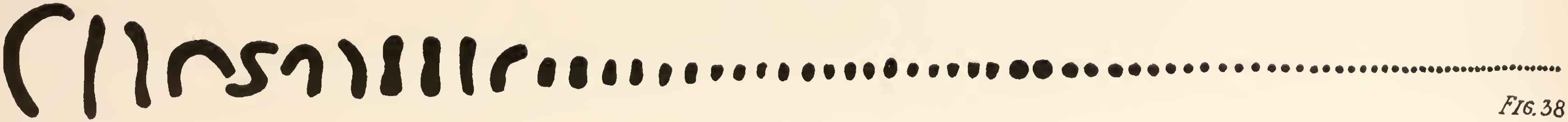

FIG. 38

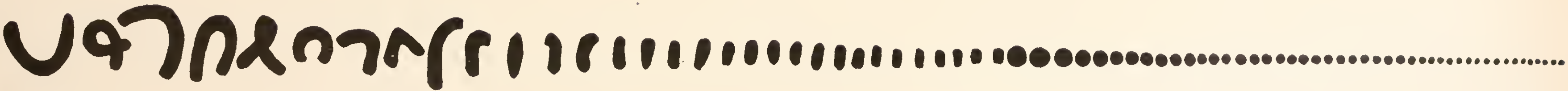

FIG. 39

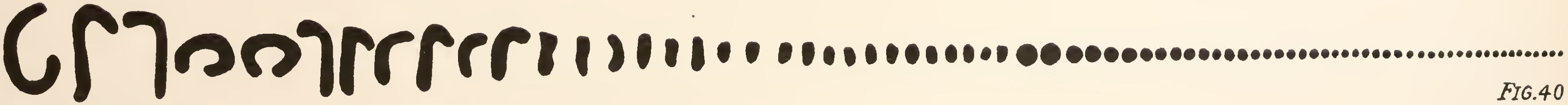

FIG. 40

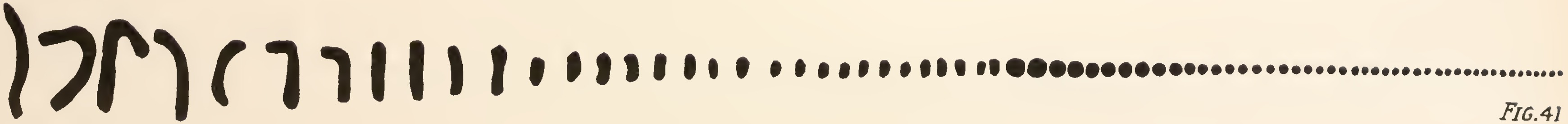

FIG. 41

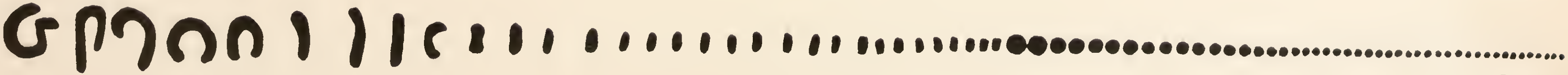

FIG. 42

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PLATE 8.

FIGS. 43 to 48. Chromosomes from the amnion cells of males artificially arranged in pairs according to their size. 38Z, 38Z indicate what are probably the sex-chromosomes. Numbers 37 to 1 are probably autosomes.

FIG. 43. Chromosomes from an early metaphase shown at Fig. 4.

FIG. 44. Chromosomes from an earlier metaphase shown at Fig. 7.

FIG. 45. Chromosomes from a metaphase shown at Fig. 3.

FIG. 46. Chromosomes from a prophase shown at Fig. 1.

FIG. 47. Chromosomes from a late metaphase shown at Fig. 6.

FIG. 48. Chromosomes from an earlier prophase shown at Fig. 2.

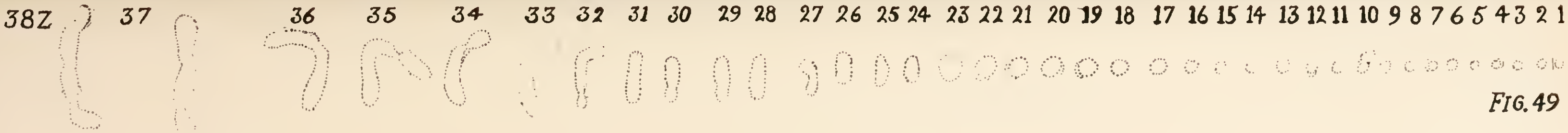
38Z
37
36
35
34
33
32
31
30
29
28
27
26
25
24
23
22
21
20
19
18
17
16
15
14
13
12
11
10
9
8
7
6
5
4
3
2
1

FIG. 49

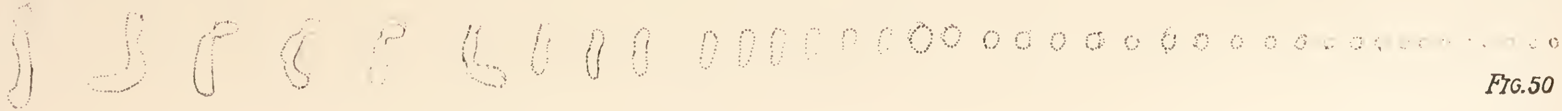

FIG. 50

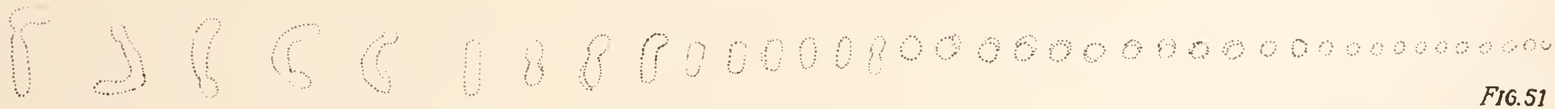

FIG. 51

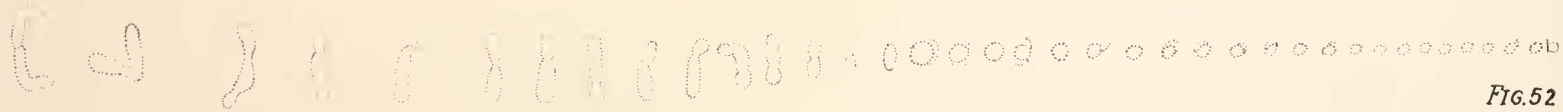

FIG. 52

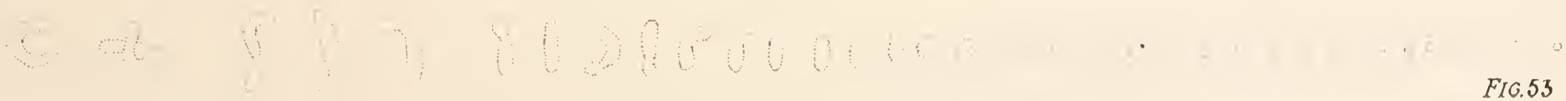

FIG. 53

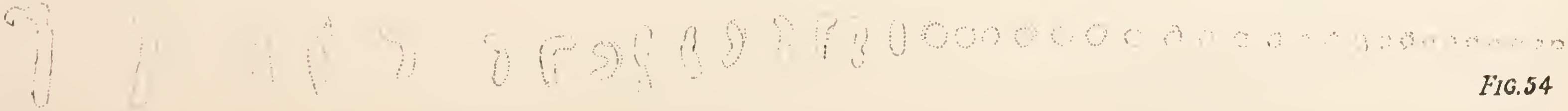

FIG. 54

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PLATE 9.

FIGS. 49 to 54. Chromosomes from first spermatocytes from smear preparations. The Z bivalent chromosome is numbered 38Z. The autosomal bivalents from 37 to 1. All the cells are in the prophase stage.

FIG. 49. Chromosomes from cell shown at Fig. 24.

FIG. 50. Chromosomes from cell shown at Fig. 27.

FIG. 51. Chromosomes from a cell shown at Fig. 29.

FIG. 52. Chromosomes from a cell shown at Fig. 25.

FIG. 53. Chromosomes from a cell shown at Fig. 28.

FIG. 54. Chromosomes from a cell shown at Fig. 26.

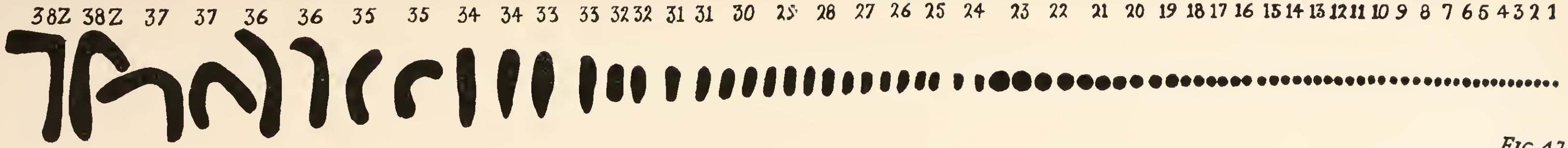
38Z 38Z 37 37 36 36 35 35 34 34 33 33 32 32 31 31 30 29 28 27 26 25 24 23 22 21 20 19 18 17 16 15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

FIG. 43

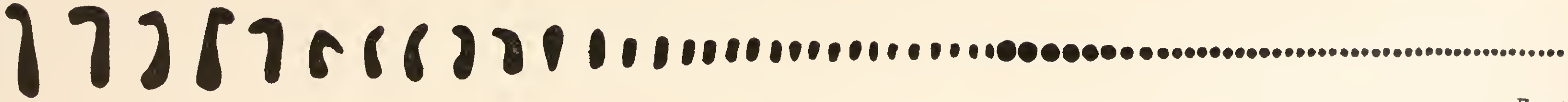

FIG. 44

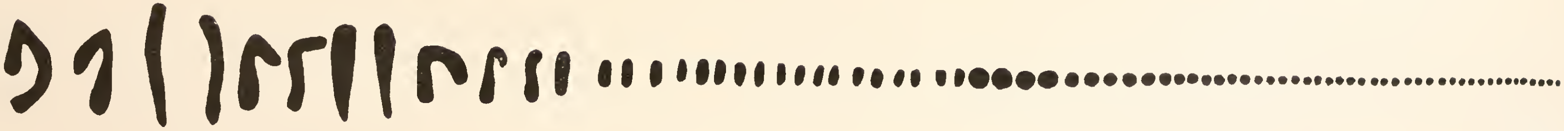

FIG. 45

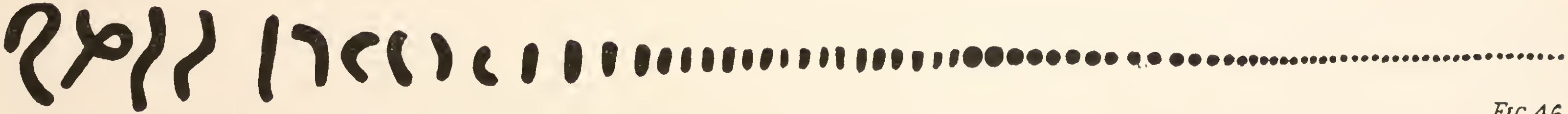

FIG. 46

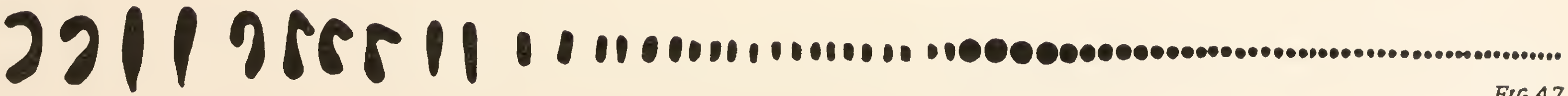

FIG. 47

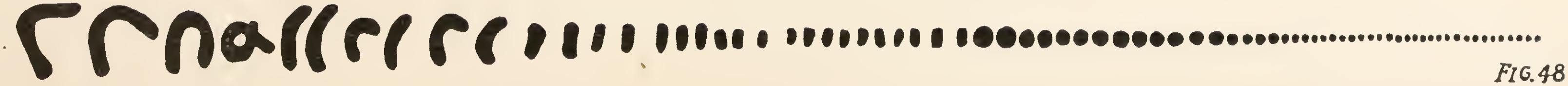

FIG. 48

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Plate 10.

Figs. 35 and 36. Scheme for sex-determination and sex-linkage in the Indian runner duck.

A indicates autosomes.

Z (double lined) indicates sex-chromosome carrying a dominant sex-linked gene, also carrying a preponderance of male-tendency genes.

Z (single lined) indicates sex-chromosome carrying a recessive sex-linked gene, etc.

W indicates the large sex-chromosome carrying female-tendency genes only.

w indicates the smaller sex-chromosome which carries likewise female-tendency genes only.

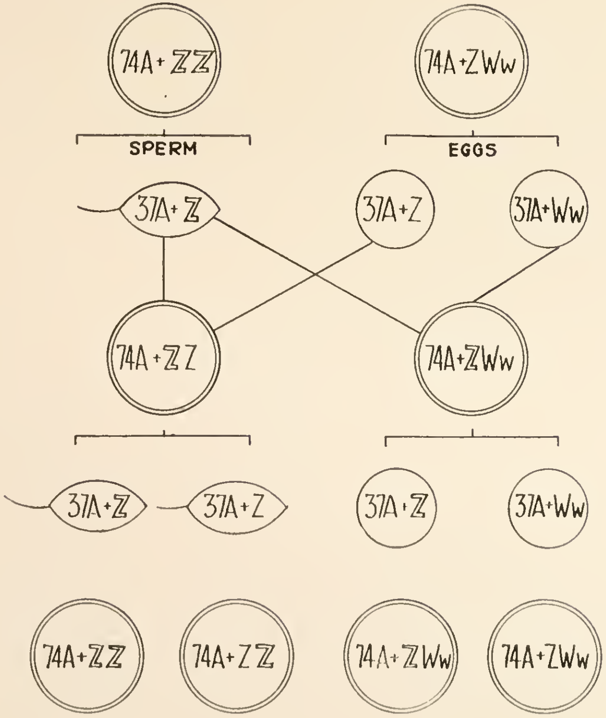
74A+ℤℤ
74A+ZWw
SPERM
EGGS
37A+ℤ
37A+Z
37A+Ww
74A+ℤZ
74A+ℤWw
37A+ℤ
37A+Z
37A+ℤ
37A+Ww
74A+ℤℤ
74A+Zℤ
74A+ℤWw
74A+ZWw

FIG. 56

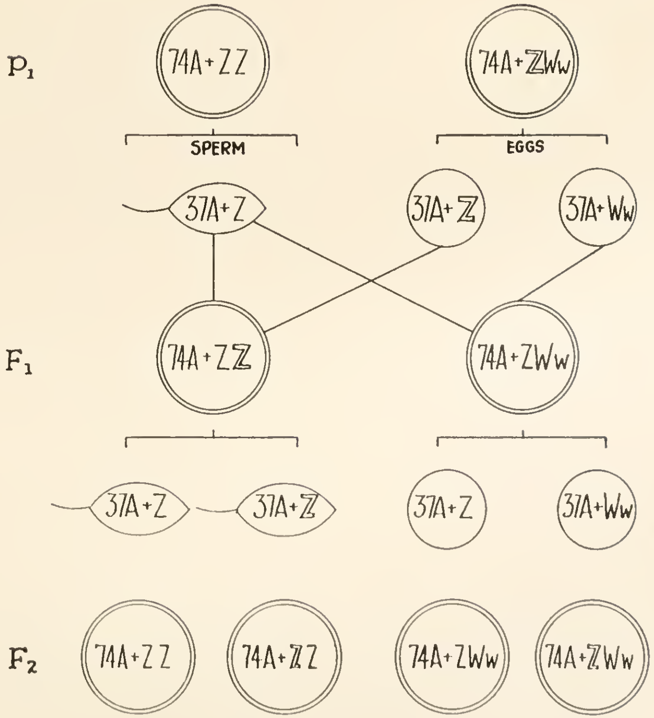
P1
F1
F2
74A+ZZ
74A+ℤWw
SPERM
EGGS
37A+Z
37A+ℤ
37A+Ww
74A+Zℤ
74A+ZWw
37A+Z
37A+ℤ
37A+Z
37A+Ww
74A+ZZ
74A+ℤZ
74A+ZWw
74A+ℤWw

FIG. 55

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DEFECTIVE *PLUTEUS* LARVÆ FROM ISOLATED BLASTOMERES OF *ARBACIA* AND *ECHINARACHNIUS*.

HAROLD H. PLOUGH,

Department of Biology, Amherst College and The Marine Biological Laboratory.

Organization in the Sea Urchin's Egg.

Ever since Driesch in 1891 announced that a single blastomere of the two cell stage of the sea-urchin's egg separated by shaking would develop into a whole larva of half size, this method has been generally used by embryologists for determining the type of organization present in various eggs at the time of the first or later cleavages. Driesch's conclusions led to much controversy, but most investigators have come to a fairly definite agreement on the major facts to be learned from this field of investigation, and have turned to other problems. The recent important work of Von Ubisch (1925) has once more drawn attention to the organization of the sea-urchin egg previous to cleavage, and has made it necessary to revise certain accepted ideas. The data which I shall present appear to be of significance in this connection, and even though at this late date it would seem impossible to add anything to the facts regarding the development of isolated blastomeres of sea-urchin eggs, this will be my excuse for briefly reviewing the evidence.

In 1900 and in 1906 Driesch repeated and amplified his earlier work and stated his conclusion that in the sea urchin egg there was no evidence of differentiation at any cleavage stage, and that the egg was an harmonic equi-potential system, each cell of which might give rise to any part of the whole larva. In 1901 Boveri studied the normal development and later isolated blastomeres, and concluded that there was an entoblast zone below the equator of the egg and at right angles to its polar axis (colored red in *Paracentrotus*), without some portion of which an isolated blastomere would not develop an archenteron. He believed that the first two cleavage planes cut this differentiated zone at right angles, and that the first cleavage plane marked the median plane of the larva. In support of this idea he pointed to the fact brought out by Driesch himself that the isolated animal half of the transversely divided eight cell stage seldom gastrulated, while the isolated basal half (vegetative portion) more often developed normally. Driesch did not accept this interpretation, and in 1906 described the development of an egg incompletely separated in the two cell stage but differentiating as one individual. Here he found that the skeletal rudiments appeared in the descendants of one only of the original cells, and he concluded therefore that the first cleavage plane cut at right angles to the median plane of the larva, and that, contrary to Boveri, the egg was first divided into animal and vegetative portions.

Here the matter has stood up to the present. The majority opinion has accepted Boveri's evidence, as shown by Conklin's summing up of the situation (1924, page 586): "It is plain that there is a differential distribution of egg substances to the cleavage cells from the animal to the vegetative poles, though no differentiation of cells in any cross axis can be detected until much later." E. B. Wilson (1925, page 1067) said: "These facts (*e.g.*, of Boveri) demonstrate that the sea-urchin egg is no more isotropic than that of the mollusk or annelid."

The experiments of Von Ubisch mentioned above prove that both Driesch and Boveri were right with respect to the relation of first cleavage plane and the median plane of the embryo, yet in spite of this are in no sense incompatible with Boveri's idea of a differential organization of the egg at right angles to the initial egg axis. This investigator has succeeded, where Boveri failed, in staining one or more of the blastomeres in dividing eggs of *Echinus* and *Echinocyanus* and then noting the portions of the larvæ which are marked with the dye. By this method he has demonstrated quite conclusively that there is no constant relation between the first cleavage plane and the median plane of the larva, since the stained area may appear in any region of the pluteus. Thus larvæ have been found which differentiated both as described by Driesch and by Boveri, and at every angle between. In spite of this fact Von Ubisch believes that his results lend support to Boveri's conception that the egg is stratified at right angles to the initial egg axis. With such a distribution of differentiated substances and the first cleavage cutting at any angle, one would expect that staining one blastomere of the two cell stage would give larvæ with the blue area in every possible sector. This Von Ubisch found. Such a result obviously is not at variance with Boveri's view, but it can hardly be called evidence in favor of it for the same might be true if there were no organization of the egg whatever. The only disproof of the latter idea would consist of the demonstration of the same sort of relation in larvæ actually developed from isolated one half blastomeres. This evidence I have secured for *Arbacia* and *Echinarachnius* as will be indicated below.

In addition to the problem of the organization of the undivided egg of the sea urchin with respect to stratification of differentiated substances, there is the added question as to a possible bilateral organization of such materials. Boveri obviously held that the right and left sides of the body of the larva were determined by the plane of the first cleavage, but Von Ubisch's work disproves this. The same criticism applies to the conclusions of Schaxel (1914) and of others who worked with isolated blastomeres. If bilaterality bears no relation to the first cleavage plane, then it must be determined in the egg before the first cleavage or at a much later stage in development. To establish the first relation, since the first cleavage cuts the egg at any angle, it would be necessary to show that isolated blastomeres formed only certain definite parts of some obviously bilateral structure like the skeleton. It seems extremely unlikely, and yet curiously enough there is good evidence for exactly this situation in at least one sea-urchin, *Paracentrotus*—the same egg, by the way, in which appears the stratified pigment layer described by Boveri. J. Runnström (1914) described the development of isolated blastomeres of this egg, and for some reason his results seem to have been largely overlooked in discussions of the subject. Quite contrary to the results of Driesch on *Echinus* and *Sphaerechinus* he found that the blastomeres of the two cell stage did not develop as whole larvæ even after the blastula stage. Instead they gave rise to partial larvæ, showing the skeleton of either the right or the left side. Such larvæ never become normal. Unfortunately data are not given to show how many blastomeres developed in this manner, nor what happened to the other member of the pair when one gave this result. Apart from this, however, Runnstrom's conclusion seems justified that there was an indication of a bilateral organization of skeleton forming material in the undivided egg of *Paracentrotus.* I have found a number of cases of the same sort in *Arbacia* and *Echinarachnius*, suggesting that in these eggs also there is already some bilaterality of skeleton forming material in the undivided egg.

The bearings of the present investigation may be shown with greater clearness by reference to a diagram. Several of the earlier writers have indicated that material which goes to form the larval skeleton seems to be localized in the four micromeres cut off from the lower quartette of cells at the fourth cleavage (sixteen cell stage). Von Ubisch has apparently confirmed this by staining these cells and finding that the material derived from them formed the larval mesenchyme. On Boveri's view this skeleton forming substance may be thought of as stratified at right angles to the initial egg axis in the undivided egg. That this is probably the case in *Arbacia* is indicated by the recent work of Harnly (1926). He reported experiments in which fertilized eggs of *Arbacia* were cut in two before the first cleavage, and he found that the nucleated portions segmented either as the dorsal, lateral or ventral hemisphere as determined by the presence or absence of micromeres. This result shows, he believes, that there is a localized equatorial area of micromere forming substance in the undivided egg below the nucleus and in the vegetative half of the egg. The later history of these fragments is not described, but we may assume that the material which will form the skeleton is located in the egg as suggested. According to Von Ubisch's evidence the first cleavage plane may cut the egg as shown in Fig. 1. *A* and *C* indicate the extremes, and *B* one of the possible planes between. According to this plan we should expect that each blastomere of *A*, if isolated would develop as a whole larva of one half size. If those of *C* are separated, however, the upper blastomere would lack the skeletal material, and one complete larva and one lacking a skeleton might be expected. Finally if one of the many eggs in which the cleavage fell between the two extremes, like *B*, was operated on, either the same result or two larvæ with defective skeletons might be looked for. The possibility of bilateral organization need not be considered for the present. In any case the results of the development of isolated blastomeres, would seem to offer conclusive evidence as to the differentiation in the sea-urchin egg in the light of the work of Von Ubisch.

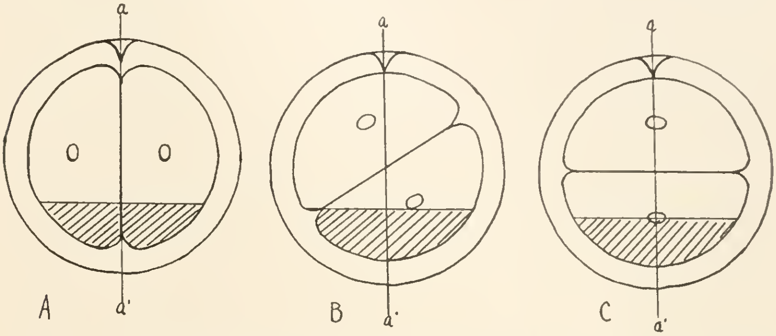

FIG. 1. Diagrams showing three possible relations of the first cleavage plane to the primary egg axis as indicated by the micropyle. The line *a–a'* is drawn through the egg axis in each case. The shaded layer suggests the possible location of the skeleton forming material.

Considered from this point of view it becomes an important matter to secure data on the later development of a large number of isolated blastomeres of sea-urchin's eggs. Such data must record the complete results and not simply the fact that complete larvæ of one half size are formed from isolated blastomeres of the two cell stage. It is obviously quite as important to know how many such blastomeres do not form complete larvæ, as to know how many do. Practically all of the earlier investigators note that a number of failures occur, but they are content with the positive demonstration. Even under the best conditions and with the greatest care in handling however, a certain number of whole eggs fail to develop normally, and this is sure to be true to an even greater extent when the cells are subjected to the handling required by the technique of separation of the blastomeres. This is true even when the earlier stages of cleavage are normal. Data of the sort suggested therefore can not be conclusive, for one can never be certain how many of the failures are due to deficiency of materials in the initial cell and how many to abnormal development resulting from the handling.

This difficulty in interpreting the results can be eliminated to a very large degree if the development of both members of the pair of blastomeres from one egg are followed, and the results recorded for single pairs rather than for groups of cells. When one or both of the blastomeres fail to develop to the stage of the ciliated larva, this pair need not be considered. When, under the same conditions and with identical handling, both blastomeres do develop to this stage the result may be considered significant evidence of organization of the undivided egg. It seems an extraordinary fact that in all of the published work on isolated blastomeres of sea-urchins there is so little in which the developmental history of each of the blastomeres derived from a single egg is known. In all of his earlier work Driesch was content to show that whole larvæ of one half size were formed from isolated one half blastomeres, and no attempt was made to prove that both blastomeres from one egg did so, nor even to indicate how many out of a large group failed to differentiate in this way. In 1906 Driesch published his first study of the "Physiology of Bilaterality," in which he did take up this question. He followed the development of both blastomeres of the two cell stage, usually two eggs at a time, in several series of experiments on *Echinus multituberculatus* and *Spærechinus*. The eggs were observed for two days only, and failures were not recorded. From this data I find that out of 9 eggs divided, only 2 gave normal one half size plutei from both blastomeres, 4 gave two "prismen" larvæ—too young to determine whether the skeleton was normal—2 from which one pluteus was complete and the other apparently incomplete, and 1 in which no skeleton appeared in either. These data are insufficient for any conclusion as to the organization of the egg along the lines indicated, yet so far as they go they suggest that both blastomeres do not necessarily develop into whole larvæ. In all of Driesch's remaining experiments, including several series reported in his 1908 paper on the same subject, the blastomeres were observed for one day only—that is to the gastrula stage—and it was assumed that they would develop into normal larvæ if they formed normal gastrulae. This assumption is quite unjustified, as my data on *Arbacia* show.

PRELIMINARY EXPERIMENTS.

Several years ago at the Marine Biological Laboratory I began experiments to determine whether in the egg of *Arbacia* both blastomeres isolated in the two cell stage would form normal half size larvæ. Each egg was handled separately, and the development was followed for at least four days. The work of the first season—1924—gave so few pairs of blastomeres each of which developed that the point could not be settled. At the same time it became apparent that one half blastomeres give rise: (1) to ciliated larvæ which either do not gastrulate or do not form a skeleton, (2) to larvæ with incomplete or partial skeletons, and (3) in a small number of cases to whole larvæ of one half size. These facts I reported briefly at the Washington Meeting of the Society of Zoölogists in 1924.

During the past two seasons at Woods Hole I have been able to extend these observations on *Arbacia*, and during 1926 I made a similar series on the egg of the sand dollar *Echinarachnius*. The latter egg is much more favorable for such work, since it is larger, has a membrane which is much less resistant, and when good lots of eggs are obtained, gives a high percentage of eggs which develop into normal plutei. It is harder to get good lots of eggs of *Echinarachnius* than of *Arbacia* at Woods Hole, and therefore the total number of blastomeres followed is less.

METHODS.

The eggs were removed from the animals into sea water, washed, and fertilized with motile sperm suspensions. During the earlier work with *Arbacia*, the membranes were removed by shaking a suspension of eggs in a small test tube for twenty seconds at an interval of one to one and a half minutes after fertilization. This violent treatment is necessary to break and remove the very tough membrane of *Arbacia*, and it generally breaks up or otherwise injures a large number of the eggs. It was later found possible to remove the membranes with great ease, either about fifteen minutes after fertilization or in the two cell stage, by sucking them up into a very fine capillary pipette with a bore about two thirds the diameter of the egg membrane. If the pipette is of the proper size, and the eggs drawn up carefully by means of an attached rubber tube held in the mouth, this method seldom injures the eggs. It was used throughout the larger part of the work. When the eggs had passed into the two cell stage, a small number were picked up with a slightly larger pipette and placed in a few drops of sea water on a clean glass slide.

The blastomeres were separated with fine glass needles used free hand under a binocular microscope. It is possible to use either one or two such needles, and with practice to acquire a high degree of skill in their use. Needles for this work must be fine enough to lie easily in the furrow between two cells, and stiff enough to allow of a small amount of pressure. Ordinarily needles which are sufficiently fine, work better if they are not too long—about ten times the diameter of the egg seemed to give the most satisfactory results. This free hand method has been used recently by a number of investigators for work in cutting eggs in preference to the more rigid and much slower micro-dissection apparatus. (Cf. especially Fry 1924, who has given a complete account of the method of making the needles and their use.) It has the advantage that many more eggs can be operated on in a given period, and with practice the control is quite as dependable. The needle is laid along the furrow between the blastomeres, and drawn gently back and forth. Usually *Arbacia* blastomeres can be separated cleanly and without injury by one or two strokes. Often the egg sticks to the needle, and rolls with it, but if this is not prolonged it seldom results in injury. In some cases the blastomeres will separate the width of the needle, but no further, remaining attached apparently by a thin bridge of protoplasm. Such eggs were usually discarded, although the blastomeres seldom come in contact again and eventually separate.

The two cell stage of *Arbacia* with the membrane removed exhibits certain differences from the normal which are of importance in separating the blastomeres. Shortly after division the two cells round out, forming two nearly perfect spheres having a very narrow area of contact. They remain in this condition for fifteen or twenty minutes, gradually flattening against each other as the next division approaches, until at the time of the second cleavage they appear as half spheres with their flattened diameters in contact. The same process occurs at each cleavage, and is apparently caused by the gradual recurrent increase in viscosity up to the time of actual division. The blastomeres must be separated during the earlier period when they are sphere shaped, since it becomes increasingly difficult later.

I have made many attempts to separate the blastomeres of Arbacia by the use of Ca-free sea water, so widely used for this purpose since the original discovery of the method by Herbst but all of these have been unsuccessful. The original Herbst solution used at Naples, sea water treated with Na-citrate, and various artificial sea waters without Ca, have uniformly failed to bring about the separation of *Arbacia* blastomeres without shaking, and this makes it impossible to keep the two blastomeres of one egg under observation. With *Echinarachnius* the method seems to be somewhat more successful, but not uniformly enough to be superior to mechanical separation.

The isolated blastomeres were picked up one by one with a capillary lip pipette, and the pairs from any one egg placed together in sea water in a round bottomed glass dish two or three centimeters in diameter with a slot ground in the center (Lefevre dishes). These dishes were more satisfactory than depression slides or other containers, since by placing the blastomeres in the slot the water can be changed without danger of sucking up the cells. The development was observed under the four millimeter objective of the compound microscope with a ten ocular.

The dishes when not under observation were kept in a moist chamber consisting of a glass evaporating dish with a cover. This was kept in the tank with running sea water constantly passing over it to maintain it at approximately the temperature of the sea water itself. The water in each dish was changed daily, actual tests showing that a negligible change in hydrogen ion concentration occurred during this period.

In general the cleavage stages of all separated blastomeres were followed and sketched. After that the embryos were observed at least once a day up to the fifth day. Several control eggs with

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the membranes removed were placed in similar dishes with each series of experiments.

EXPERIMENTS.

Summary of All Isolated Blastomeres.—In Table I. a tabular view of all of the results is given, regardless of whether or not both members of the pair of isolated blastomeres developed. All blastomeres which began cleavage normally are included. In the last three columns the figures in brackets are the percentages of the number which lived to the ciliated larva stage.

TABLE I.

(1) Number of Blastomeres Isolated.	(2) Total Number of Gastrulæ.	(3) Ciliated Larvæ with No Skeleton.	(4) Pluteus with Defective Skeleton.	(5) Complete Pluteus of ½ Size.
Arbacia, 268	121	46 (47%)	24 (24%)	28 (29%)
Echinarachnius 33	20	5 (31%)	6 (38) %	5 (31%)

The summary indicates that a large number of isolated blastomeres even when apparently uninjured, do not develop normally. Less than 50 per cent. of the *Arbacia* cells developed to the gastrula stage, although the number in the sand dollar was larger. Of the isolated cells which lived to become ciliated larvæ, 47 per cent. in *Arbacia* and 31 per cent. in the sand dollar never developed a skeleton. Doubtless a certain number of these were

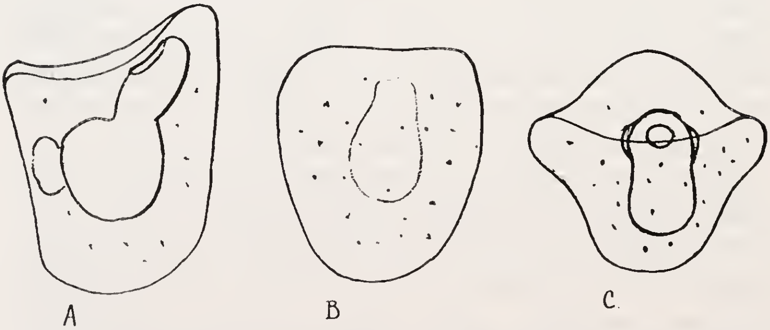

FIG. 2. Ciliated larvæ with no skeletons developed from single blastomeres of the two cell stage of *Arbacia*. Four days old. Measured and drawn free hand from living specimens to the same scale.

injured and therefore failed of normal development, but in view of the evidence given in the next table where the development of pairs from the same egg are listed, it is probable that some of them were deficient in cytoplasmic material already localized at

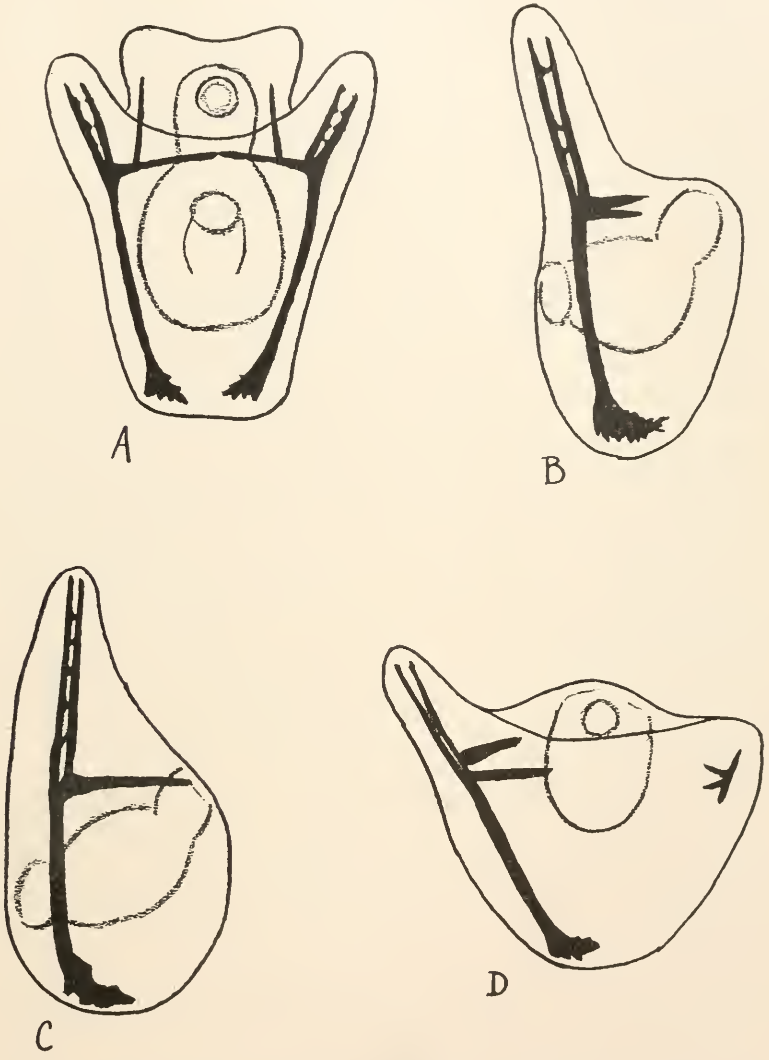

FIG. 3. Pluteus larvæ four days old developed from single blastomeres of the two cell stage of Arbacia. *A* is a larva with a complete skeleton of approximately one half size. *B*, *C*, and *D* are larvæ with defective skeletons. Measured and drawn free hand to the same scale from living specimens.

the time of the operation which later goes to form the skeleton. Many of these larvæ lived for eight days or even more, but always remained enlarged ball-like larvæ (Fig. 2). In perhaps half of these larvæ a normal tripartite gut was formed, while in the others either a small portion or none at all was differentiated. Some of these larvæ were probably derived from blastomeres like the upper one in Fig. 1 *C*.

The larvæ listed in the fourth column of Table I. are of special interest. They were briefly described in my preliminary report. All showed the development of a skeleton but this was incomplete. The commonest type was a four day pluteus with one long postoral arm and the other absent. The oral portion of the skeleton was usually missing entirely. Fig. 3 shows several such larvæ compared with a complete one half size larva of the same stage. Such larvæ of *Paracentrotus* have been figured by Runnström (1914) in earlier stages. It seems impossible to escape the impression that such larvæ are derived from one of the blastomeres of an egg divided like *B* in Fig. 1.

The larvæ listed in the last column of Table I. were normal but of approximately one half size (Fig. 3). They were complete in every observable detail, and were usually as viable as normal control plutei. Such larvæ are probably derived from cells divided along the egg axis (Fig. 1 *A*). It was observed that isolated blastomeres developed at a slower rate than normal control eggs. The first cleavage appeared fifteen to twenty minutes after the second in the normal egg, and the gastrulæ were sometimes two hours or more behind.

Finally it should be stated that I have never found larvæ with defective skeletons among the normal controls unless these were subjected to abnormal treatment. Shaken eggs sometimes give as many as 15 per cent. of abnormal skeletons, but never eggs handled as indicated. Almost every group of control eggs shows a few larvæ which do not gastrulate, or which do not form skeletons, but these seldom live more than two days and usually less.

Pairs of Blastomeres from the Same Egg.

If the results summarized above seem inconclusive because of the large number of blastomeres which fail to develop to the pluteus stage, it seems possible to attain to a fair degree of certainty by limiting our consideration to those eggs only of which both blastomeres developed to the stage of a ciliated larva. These data I have tabulated in Table II.

TABLE II.

SHOWING TYPES OF LARVÆ RESULTING FROM THE TWO BLASTOMERES OF THE SAME EGG, WHEN BOTH LIVE TO THE CILIATED STAGE.

(1) Number of eggs Separated	Pairs of Separated Blastomeres Gave rise to					
	(2)	(3)	(4)	(5)	(6)	(7)
	2 Ciliated Larvæ with No Skeleton.	1 Larva No skeleton and 1 Complete Pluteus.	1 Larva No skeleton 1 Defective Pluteus.	2 Defective Plutei.	1 Defective Pluteus, 1 Complete Pluteus.	2 Complete Plutei.
Arbacia, 18 (100 %)	8 (44%)	5 (28%)	2	1	1	1
			17%		11%	
Echinarachnius, 9 (100%)	1 (11%)	3 (33%)	1	3	1	0
			45%		11%	
Representing probable relation of plane of first cleavage to egg axis as in:	?	Figure 1 C	Figure 1 B		Figure 1 A	

Table II. is largely self-explanatory. The total number of eggs of which both blastomeres developed is only 7 per cent. of the total number of blastomeres followed in *Arbacia*, but is 54 per cent. in the sand dollar. When good eggs of the sand dollar can be secured it is obviously a much more favorable species for such work. The same fact appears in column (2), since in 44 per cent. of the eggs of *Arbacia* which gave rise to two larvæ no skeleton developed in either, while only one out of nine, or 11 per cent. gave this result in the sand dollar. This fact introduces a source of error in the interpretation of columns (3) and (4), for it is not certain that some of the larvæ with no skeleton here might not have possessed skeleton forming material. This possible error is not serious, however, as an examination of the remaining data will show.

A study of the remaining columns in the table reveals three fairly striking facts. First, the number of pairs of blastomeres each of which form complete larvæ is very small indeed. I have found only one out of eighteen eggs which gave this result in *Arbacia*, and none out of nine in the sand dollar, although the one recorded in column (6) showed only a slight defect in one arm of one of the larvæ. Second, among such pairs larvæ with defective skeletons are fairly common, but occur most often in pairs, column (5). Probably the error shown by column (2) has prevented some of those listed in column (4) from appearing in (5). Third, there is a fairly large group of pairs of which one member formed one complete half size pluteus and the other a larva with no skeleton, column (3).

For the purpose of our analysis then there would appear to be three significant groups of eggs. While the actual numbers are small they form a selected group from a very much larger number, which as shown above tends to suggest the same result. The first group consists of the small number of eggs each of whose blastomeres formed perfect plutei, the second of eggs each blastomere of which formed an incomplete pluteus, and the third of eggs one blastomere of which formed a perfect pluteus and the other a larva with no skeleton. It is obvious that these three main groups correspond in general with the three possible directions of the first cleavage plane with reference to the initial egg axis as suggested by the work of Von Ubisch, and as indicated in diagrams *A*, *B*, and *C* in Fig. 1. This interpretation is noted in the last line of Table II. Not only are these three groups found, but in the sand dollar eggs at least the percentages of eggs which fall in them correspond with expectation. The second group should be the largest, and the first the smallest, as in fact they are.

Defective Plutei and the Evidence for Bilaterality of Skeleton-Forming Material.

A study of the larvæ with defective skeletons not only substantiates the facts stated above, but suggests in addition a certain amount of bilateral organization of the skeleton-forming material which is apparently already localized in the vegetative half of the egg at right angles to the initial egg axis. All of the larvæ listed in column (5), Table II., showed defective skeletons. Two sets of these—the *Arbacia* pair and one of the pairs from an *Echinarachnius* egg—were larvæ of the one armed type illustrated in Fig. 3. The other two pairs of *Echinarachnius* larvæ showed skeletons which were clearly complementary, that is the one member showed the parts which the other lacked. These two pairs of larvæ are shown at the age of four days in Fig. 4, *A* and

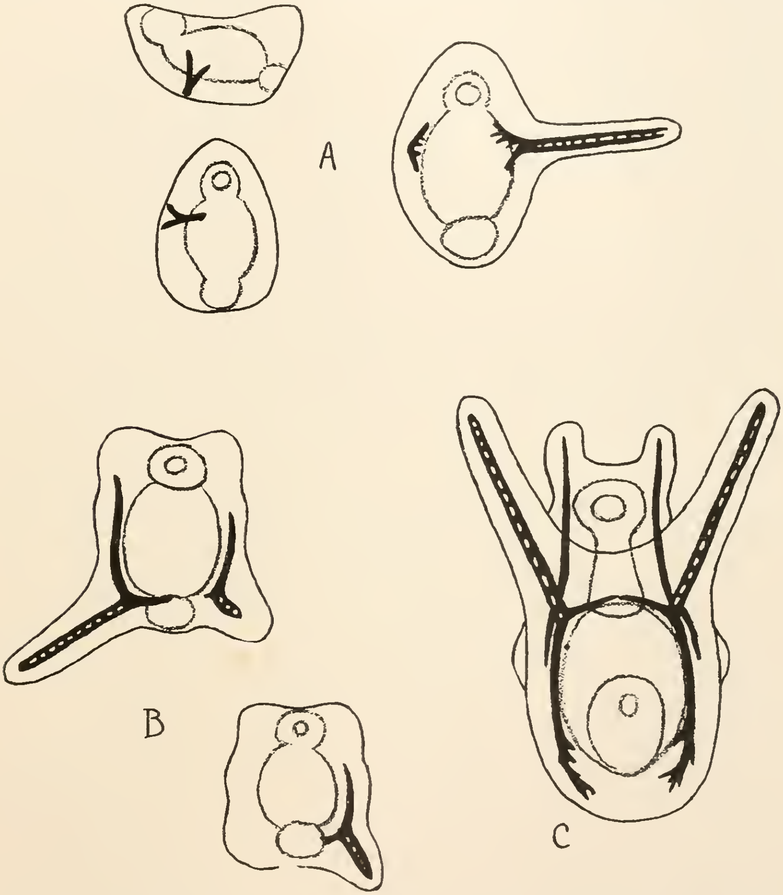

FIG. 4. Pluteus larvæ of *Echinarachnius* four days old. A shows two larvæ developed from the two isolated blastomeres of the two cell stage of the same egg, the smaller one shown from the side and the top, the larger from the top only. *B* shows a similar pair from the top only. *C* shows a normal control larva of the same age, aboral view. Measured and drawn free hand to the same scale from living specimens.

B, compared with a normal control larva of the same age in *C*. All the defective larvæ had a complete tripartite gut like the normal. In the *A* pair one shows a skeleton like the control but with the large *left* postoral arm lacking, while the other has only a rudiment of a spicule on the left side—the *right* side completely lacking. These larvæ were followed for two days longer and the latter never developed any farther, while the former never replaced the defect. In *B* much the same result is shown, perhaps more distinctly, but rather more skeleton appeared in the smaller larva. As indicated above Runnström (1914) described such larvæ in earlier stages as occurring regularly in *Paracentrotus*, and suggested that the undivided egg showed a bilateral organization. The finding of these plutei in *Arbacia* and the sand dollar constitutes very clear proof that a localization of skeleton forming material has taken place in the undivided egg, and that this may be divided unequally by the first cleavage plane. It also indicates a bilateral organization of this material. It seems quite probable that the reason that this bilaterality is not more often evidenced is that blastomeres with a marked deficiency of skeleton-forming material often fail to develop the skeleton rudiment at all.

It may be asked in view of this interpretation how it happens that two complete plutei ever develop from both blastomeres of one egg. To this it may be answered that my data indicate that such cases are extremely rare in *Arbacia* and the sand dollar. When the stratified material is about equally divided apparently the initial bilaterality may be reorganized from the start. That such reorganization may occur infrequently even in later stages seems to be proved by the history of one *Arbacia* blastomere, in which the steps were noted with great clearness.

In the case illustrated in Fig. 5 one isolated blastomere began its development as a partial one, but later a complete but small skeleton was regenerated—or postgenerated. In this case both blastomeres of the egg went through the normal one half cleavage, becoming rounded up at the blastula stage. Two one-half-size gastrulæ were formed, but one never became motile and soon died. The other apparently formed a single triradiate spicule and developed at the end of two days a half skeleton with a single postoral arm, and the crossbars as indicated in the sketch. On the third day the skeletal material had extended over to the other side and a second postoral arm had appeared. The oral part of the skeleton was defective. By the fourth day a skeleton with all parts represented, but abnormally massive, and of rather less than the usual size was formed.

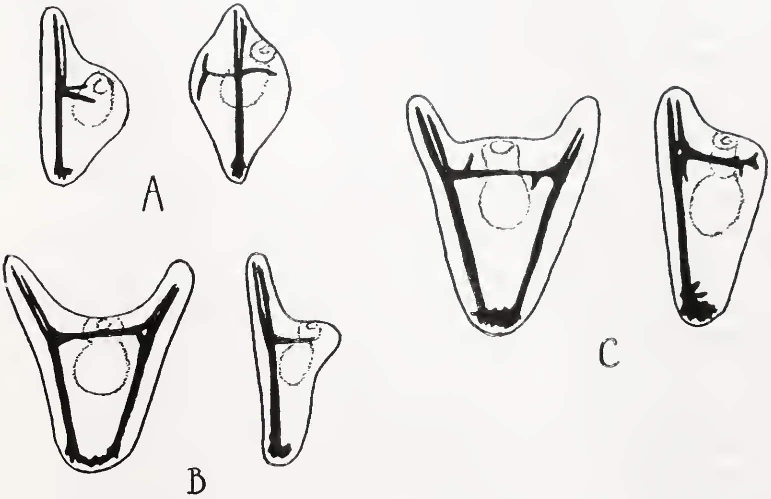

FIG. 5. Successive drawings of a pluteus larva developed from a single blastomere of the two cell stage of *Arbacia*. The skeleton was first one-sided, but later all except the oral portion was regenerated. *A* two days old, *B* three days old, *C* five days old. An aboral and a side view are shown in each case. Measured and drawn free hand to the same scale from the living specimen.

CLEAVAGE OF ISOLATED BLASTOMERES.

The work of Driesch, of Morgan, and others indicated that the cleavage of isolated blastomeres of *Echinus* and *Sphærechinus* was that of half embryos. Each blastomere divided into four equal cells, and at what corresponds with the fourth cleavage of the original egg gave rise to two micromeres, two macromeres and four mesomeres, just as they would have done in the normal egg. They later formed an open blastula with half the normal number of cells, which eventually rounded up. Since in the cleavage of *Echinarachnius* there are no clearly distinct micromeres, it is impossible to compare its cleavage with that of the others. The cleavage of the one half blastomeres of *Arbacia* was followed, but because of the difficulty of being certain of all the cells when rapid cleavage was going on, and when a number of eggs are under observation at the same time, I have accurate records on only about twenty-four blastomeres of this type. Fifteen of these developed as stated above for *Echinus*, namely giving off two micromeres at the third cleavage (normal fourth). The others appeared to have formed no micromeres at all—rather eight equal cells—though two may have had one only. Only eight of the total number developed to the pluteus stage, a number far too small from which to draw positive conclusions. From half eggs showing two micromeres there developed larvæ both with and without a skeleton, and in two cases a larva with a skeleton developed from blastomeres in the cleavage of which no micromeres were observed. So far as they go the data suggest that there is no essential relation between the presence of micromeres in artificially divided eggs and the development of a skeleton. Yet Von Ubisch has shown that the skeletal material is normally passed out into the micromeres, and Harnly's work apparently indicates that when the vegetative portion of the undivided egg is cut off, micromeres do not develop. On the other hand Tennent and Taylor, working on another egg cut before fertilization, did get normal larvæ from fragments which formed no micromeres. It seems probable that normally the pattern of cleavage is determined by the first cleavage plane, and the skeleton forming material goes as usual into the micromeres. When the first two blastomeres are severed this pattern is ordinarily continued, but it may sometimes be disturbed. Whether it is or not apparently has no influence on the development of a skeleton, since that in *Arbacia* at least appears to depend on the presence or absence of skeleton forming material in either or both of the separated blastomeres.

Since this paper was written the more complete account of the work on development of fragments of the unfertilized egg of *Lytechinus* by Taylor, Tennent and Whitaker (1926) has appeared. Their work convincingly proves that in this egg micromere forming substance is not differentiated before fertilization. Whether their disagreement with the findings of Harnly is due to differences in behavior between *Lytechinus* and *Arbacia*, future work may show. In any case their observations as to the variability in the number of micromeres agree with my own in the development of the severed blastomeres of the two cell stage of *Arbacia*. If the eggs are similar, it would seem to be indicated clearly that the localization of material which later forms the skeleton occurs between fertilization and the cutting through of the first cleavage.

DIRECT DETERMINATION OF THE RELATION OF THE EGG AXIS TO THE FIRST CLEAVAGE PLANE.

The interpretation indicated by the data presented above, namely that there is a stratified layer of skeleton forming material at right angles to the egg axis which may be cut at any angle by the first cleavage and unequally distributed to the first two blastomeres, is obviously susceptible of a direct test. It is possible to determine the egg axis, using the micropyle as a pointer, by the use of a suspension of Chinese ink in the sea water. The angle which the first cleavage plane makes with this axis can then be noted, the membrane removed, and the blastomeres separated. In practice however this is an exceedingly difficult and time-consuming operation. In *Arbacia* at least it is often difficult to make out the micropyle with certainty, and the large number of blastomeres which fail to develop to the larval stage after membrane removal and separation usually make the laborious determination valueless. I have not been able to carry it out in enough cases with *Arbacia* to make the results of any significance. The method was not tried with the eggs of *Echinarachnius*, which appears to be a more favorable species for this work. In future work I hope to make this determination in a larger number of cases.

The relation of the egg axis to skeleton forming or other material localized in the egg might be tested in the same way by Von Ubisch's method also. The primary axis might be determined, and with this actually in view, a sector of the egg at right angles to it might be stained with the purple dye. Von Ubisch has apparently not made this determination, probably because of the difficulties involved.

SUMMARY AND CONCLUSIONS.

1. The developmental history of more than three hundred blastomeres of *Arbacia* and *Echinarachnius* isolated in the two cell stage and followed individually for at least four days is recorded.

2. About forty per cent. of these blastomeres form ciliated larvæ with a complete tripartite gut, but these larvæ fall in three classes with respect to the skeleton; (*a*) those which never develop a skeleton, (*b*) those in which the skeleton is defective or partial, (*c*) those with a complete pluteus skeleton of one half size.

3. When the pairs of blastomeres from the same egg both of which developed to the larval stage are considered alone, it is found that they fall into three significant groups: (*a*) a very small number of pairs from which developed two plutei with perfect skeletons of one half size, (*b*), a much larger number from which developed two plutei each with a skeleton which was incomplete, (*c*), a group which formed one perfect pluteus and one larva without a skeleton.

4. These facts appear to prove that there is already localized at the time of the first cleavage in the eggs of *Arbacia* and *Echinarachnius* a layer of skeleton forming material at right angles to the primary egg axis, and in the vegetative half of the egg, which may be cut at any angle by the first cleavage plane and unequally distributed to the first two blastomeres.

5. The eggs of these two sea-urchins are thus shown to be (to use the words of Professor Wilson) "no more isotropic than those of the mollusk or annelid." It seems probable that this is true for other sea-urchin eggs as well.

6. In some cases the two incomplete skeletons of a pair of blastomeres from the same egg are complementary. This suggests further a certain amount of bilateral organization of the skeleton forming material already localized in the egg at the time of the first cleavage. This idea receives support from the observations of Runnström on *Paracentrotus*.

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THE NATURAL GROUPING OF THE BREMIDÆ (BOMBIDÆ) WITH SPECIAL REFERENCE TO BIOLOGICAL CHARACTERS.[1]

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In the introductory pages of his rather negativistic treatise on the psychobiology of bumblebees, Wagner ('06), after calling attention to the immense taxonomic importance of biological characters in the case of spiders, cites—by way of contrast—several examples of the disconcerting polymorphism in the coloration and habits of bumblebees and comes to the following pessimistic conclusion:[2] "These are the facts which compel us from the very beginning to give up all hope of finding fixed and unalterable characters in the taxonomy of bumblebees for the elucidation of the biology of these insects, and *vice versa*, of deriving suitable data from their biology for taxonomic purposes."

As we shall see later, this statement of Wagner ('06), as so many other of his assertions (cf. von Buttel-Reepen, '07, '14; Wheeler, '19; and Plath, '23*b*, '24), is based upon superficial observations and is wholly untenable. During the past fifty years, several important attempts, most of which antedate Wagner's ('06) work, have been made to subdivide the Bremidæ into natural groups. These subdivisions have been based either upon (1) coloration, (2) structure, or (3) habits, and in some cases on two or all three of these.

In this paper the writer wishes to discuss in detail those attempts to subdivide the Bremidæ into natural groups which have been based primarily upon biological characters, and to subject these latter to a critical examination in the light of extensive biological observations on some of our American species. Before doing so, it seems desirable however to review briefly several efforts at grouping which have been made on the basis of (1) coloration and (2) structure.

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[1] Contributions from the Entomological Laboratory of the Bussey Institution, Harvard University, No. 247.

[2] "Dies sind die Tatsachen, welche uns von allem Anfange an zwingen, jede Hoffnung darauf aufzugeben, in der Systematik der Hummeln feste und unveränderliche Züge für die Aufklärung der Biologie dieser Insekten zu finden und umgekehrt aus dem biologischen Kriterium geeignete Angaben für die Systematik derselben . . . zu schöpfen."

Grouping on the Basis of Coloration.

The Austrian entomologist von Dalla Torre ('82) is apparently the only one who attempted to subdivide the Bremidæ on the basis of coloration. However, since many species of the genus *Bremus* (*Bombus*) which are not closely related show very striking similarities in coloration, *e.g.*, *Bremus americanorum* [3] and *Bremus terricola*, this method of grouping is a very artificial one and for this reason has not been favorably received by other workers. In this connection the present writer would like to confess that he himself, not long ago (cf. Plath '22*a*, pp. 40–41), made a similar mistake with respect to the genus *Psithyrus*.

Grouping on the Basis of Structure.

There have been several attempts made to group the Bremidæ on the basis of structure. The first of these was by the Russian General Radowszkowski ('84) who divided the genus *Bremus* into eleven groups. This scheme of grouping was later somewhat modified by Franklin ('12/'13) in his "Bombidæ of the New World," in which the then-known, eighty-five American species of the genus *Bremus* are divided into seven groups, a procedure which has been followed by other American workers. This method of grouping has been further extended by Franklin ('12/'13) to the American species of the genus *Psithyrus* which he divides into three groups.

Another scheme of grouping which has much in common with Radowszkowski's ('84) is that of Vogt ('11) who divides the genus *Bremus* into nine subgenera, which Ball ('14) and Krüger ('16 and '20) later increased to ten and thirteen respectively. The last-named author, in addition to making a few modifications in the nine subgenera established by Vogt ('11), has divided the genus *Bremus* into two sections, on the basis of the presence or absence of a spinous projection on the posterior, distal angle of the metatarsus of the middle legs. This character may prove of considerable importance in determining the genetic relationship of the various species of bumblebees, since, as we shall see later, it seems to go hand in hand with certain fundamental differences in the method by which these insects feed their larvæ.

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[3] Dr. Joseph Bequaert and the writer have recently studied De Geer's description and figure of *Bremus pennsylvanicus*, and, like Dr. T. H. Frison ('23), have come to the conclusion that it is better to use the name *americanorum* until the type specimen of De Geer is located.

Another independent subdivision of the non-parasitic bumblebees on the basis of structure is that by Robertson ('03), who removed certain species from the genus *Bremus* (*Bombus*) and erected the genus *Bombias*, chiefly on the basis of the size and position of the ocelli. This innovation does not seem to have found favor with European workers, but *Bombias*, either as genus or subgenus, is in common use in the United States.

Among the attempts to divide the Bremidæ into natural groups on the basis of structure, we may also include that of Friese and von Wagner ('10), who constructed a "Stammbaum" which is intended to show the probable genetic relationship of the fifteen species of *Bremus* occurring in Germany. This scheme of grouping will, no doubt, have to be considerably modified, since it is not in harmony with certain fundamental biological and structural characters which have been pointed out by Sladen ('99 and 12) and Krüger ('16 and '20).

Grouping on the Basis of Habits.

The first one who used biological characters for subdividing the Bremidæ was the English entomologist Smith ('76). In the second edition of his "Catalogue of British Bees," he divides the English species of the genus *Bremus* into Surface-builders (Section I.) and Underground-builders (Section II.). While this method of subdividing the Bremidæ may be of some value to the amateur naturalist, it is of little importance for taxonomic purposes, since certain species of bumblebees, *e.g.*, *Bremus vagans*, make use of many diverse nesting sites: underground, on the surface, in stone walls, hollow trees, birds' nests, attics, fur coats, etc.

A more promising and dependable biological character for dividing the Bremidæ into natural groups was discovered by the late F. W. L. Sladen ('99) who called attention to the fact that the various English species of the genus *Bremus* employ two very distinct methods of feeding their larvæ. Since this short, but epoch-making paper of Sladen ('99) has been overlooked by several workers, and since it forms the chief basis for the present discussion, it seems advisable to quote the two following paragraphs from it.

"Taking nests of humble-bees and keeping them under observation in specially constructed hives has been a hobby of mine for some years, and it is astonishing what a quantity of interesting information one can gather in this way about the habits and life-history of the dozen or so species that have been recorded from this country, several of which are very common and familiar to every one. It appears that each species has habits and proclivities more or less peculiar to itself, and these, if they could be accurately observed and recorded, would help very much in the systematic arrangement of the species, which in this interesting genus is unusually difficult, owing to the lack of easily recognisable structural differences, and to the little reliance that can be placed on colouring.

"As a result of taking a number of nests it appears that most of the *Bombi* found in this country may be separated into two groups, on what seems to be a rather important difference in the manner of raising their young. These groups may be conveniently named (1) the '*pouch-makers*' and (2) the '*pollen-storers*.' The *pouch-makers* form little pockets or pouches of wax at the side of a wax-covered mass of growing larvæ, into which the workers drop the pellets of pollen direct from their hind tibiæ on the return to the nest from the fields. The *pollen-storers*, on the contrary, store the newly gathered pollen in waxen cells specially made for the purpose, or in old cocoons specially set apart to receive it, from which it is taken and given to the larvæ through the mouths of the nurse-bees as required."

Thirteen years later, Sladen ('12, pp. 40–44, 152–153), in addition to elaborating the foregoing plan in general, changed the term *Pouch-makers* to *Pocket makers*, and further subdivided the latter group into *Pollen-primers* and *Carder-bees*. However, shortly after this revision had appeared in print, Sladen ('12, pp. 274–275) discovered that the character on the basis of which he had established the *Pollen-primers* is not a dependable one and hence inserted the following paragraph among his "Additional Notes": "*B. latreillellus* was considered to be a pollen-primer because pollen was found under the eggs in a nest in an advanced stage examined in 1911. But in a nest in an earlier stage kept under observation in 1912 the eggs were laid in cells that contained no pollen, although in at least one case pollen was put into the cell and removed before the eggs were laid. Should future investigation show that *latreillellus* is a pollen-primer only under abnormal conditions, a better name for the group, consisting or *ruderatus*, *hortorum*, *latreillellus* and *distinguendus*, would be 'Long-faced Humble-bees'; the term 'pollen-primers' could then be restricted to *ruderatus* and *hortorum*. *Latreillellus* and *distinguendus* are not closely related to *ruderatus* and *hortorum*."

For several summers the present writer has devoted most of his time to the study of the biology of bumblebees, and has had occasion to examine the nests of about 200 *Bremus* colonies belonging to the following North American species: *affinis*, *americanorum*, *bimaculatus*, *fervidus*, *impatiens*, *perplexus*, *occidentalis*, *separatus*, *ternarius*, *terricola*, *and vagans*. This material furnished an excellent opportunity to test the soundness of Sladen's ('99 and '12) classification, as applied to four of our seven American groups. The results of this investigation show that Sladen's ('99 and '12) classification provides an excellent basis for further taxonomic and phylogenetic work, but that certain details of his scheme will have to be modified.

As already stated, Sladen ('12) divides the English Bremidæ into two main groups, the *Pollen-storers* and the *Pocket-makers*. In regard to the last-named group Sladen ('12, p. 44) says: 'When the usual receptacles for pollen employed by a particular species are not available, it may adopt those employed by others. Thus in a strong nest of *B. agrorum*, one of the pocket-making species that I had under observation in 1910, the workers, during a period when there were no growing larvæ and consequently no pockets for pollen, dropped all the pollen they brought home into a special waxen cell they had constructed, like *terrestris*, on the top of some cocoons. Also a colony of *B. hortorum*, another pocket-maker, being in an advanced stage, and having no growing larvæ, placed pollen in the cocoons vacated by the young queens, but only lined the interior of the cocoons with it."

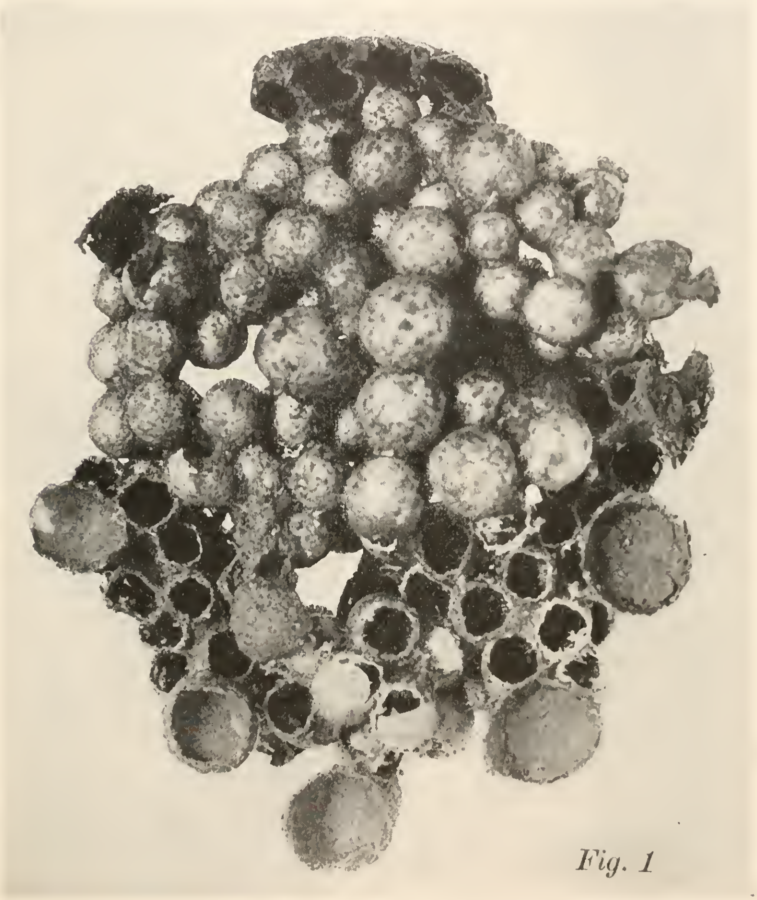

FIG. 1. Comb of *Bremus fervidus* with five pollen bowls on the lower half of the periphery. Natural size.

In this connection the present writer would like to call attention to the fact that *Bremus americanorum* (cf. Franklin, '12/'13 p. 405) and *Bremus fervidus*, two of our American *Pocket*-

makers, not only store pollen under the conditions specified by Sladen ('12, p. 44), but that toward the end of the summer—at the height of brood-rearing—prosperous colonies of these two species store considerable quantities of pollen, in some cases in shallow waxen bowls which are usually constructed near the periphery of the comb. (cf. Fig. 1). From these facts it is evident that Sladen's ('99 and '12) term *Pollen-storers* is of little use in any comprehensive classification of the Bremidæ.

Furthermore, in regard to certain *Pocket-makers*, *e.g.*, *B. americanorum* and *B. fervidus*, it must be pointed out that the habit of making pockets is resorted to only in the case of those larvæ which are destined to become workers, while the brood which furnishes the other two castes (queens and males) is fed by regurgitation; *i.e.*, in the same manner as are those of the non-pocket-making species, a fact to which the writer has called attention in an earlier paper (cf. Plath '23*a*, p. 339). However, since this method of feeding the male and queen larvæ is not employed—at least in the temperate regions—until toward the end of the breeding season, and since the method by which these Bremidæ feed their worker brood is quite distinctive, it seems best to retain the term *Pocket-makers*, unless future investigations show that the use of this term is impracticable.

We now come to Sladen's ('12) division of the *Pocket-makers* into *Pollen-primers* and *Carder-bees*. As already mentioned, Sladen later ('12, pp. 274–275) removed two species from the *Pollen-primers*; but, as will be seen from the following observations, the term *Pollen-primers* will have to be completely rejected as a subdivision of the *Pocket-makers*.

In the fall of 1921 the present writer (cf. Plath '22*a*, p. 34 and '22*b*, p. 195) made detailed observations on a large colony of *Bremus impatiens*, a non-pocket-making species, and in this case the egg-cells were regularly primed with pollen pellets before the workers oviposited in them.[4]

Even more unsatisfactory is the term "Long-faced Humble-bees" which Sladen ('12, p. 275) introduced later, since *Bremus mendax*, which structurally belongs to an entirely different group, and probably is a non-pocket-making species, has a longer face (cf. Krüger, '20, pp. 310 and 359) than either of the two species mentioned by Sladen ('12, p. 275).

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[4] Incidentally this observation also shows that von Buttel-Reepen's ('03, p. 35) explanation of the pollen priming habit of bumblebees is incorrect (cf. also Sladen, '12, pp. 274–275).

Equally impracticable is the term *Carder-bees* which Sladen ('12, pp. 152–153) applies to the second subdivision of the *Pocket-makers*. The collecting of nesting material, the character on which Sladen ('12, p. 17) based this group, is more or less common to all species of the genus *Bremus*, including the non-pocket-making species. However, by the rejection of the term *Carder-bees*, the writer does not wish to imply that the species which Sladen ('12, p. 152) includes under this term do not constitute a distinct group.

Having pointed out the inadequacy of Sladen's ('12) classification as applied to some of our North American Bremidæ, the writer would suggest the following changes in Sladen's ('12) scheme. The name *Marsipœa*[5] (from Greek *marsipos*, a pouch, and poiein, to make) is proposed as a substitute for Sladen's ('12) term *pocket-makers*. This change would result in a suitable name for Sladen's *pollen-storers* (the non-pocket-making species) which could then be designated as the *Amarsipœa*. For reasons already stated, it is further suggested that the terms *Pollen-primers*, *Long-faced humble-bees*, and *Carder-bees* be droped as subdivisions of the *Marsipœa*.

Concerning two species belonging to the *Amarsipœa*, Sladen ('12, p. 36) says: "The larvæ of *B. terrestris* and *lucorum* do not keep together in a compact mass, but as they begin to grow large each one acquires its own covering of wax, although they do not separate completely; the cocoons, therefore, do not form definite clusters, and are easily detached from one another." And in the next paragraph he continues: "With most of the species the skin of wax that covers each batch of larvæ is to the unaided eye unbroken, but as the larvæ grow, *B. terrestris, lucorum, and latreillellus* leave visible holes in the wax, which, when the larvæ approach full size, become large. The larvæ would now run the risk of falling out of their soft wax covering, which would mean their destruction, for a naked larva is always carried out of the nest; but they avoid this danger by enclosing themselves in a loose web of silk, doing this a day or two before they begin to spin their cocoons."

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[5] The writer here wishes to express his thanks to Professors W. G. Aurelio and W. M. Wheeler for their valuable suggestions concerning these terms.

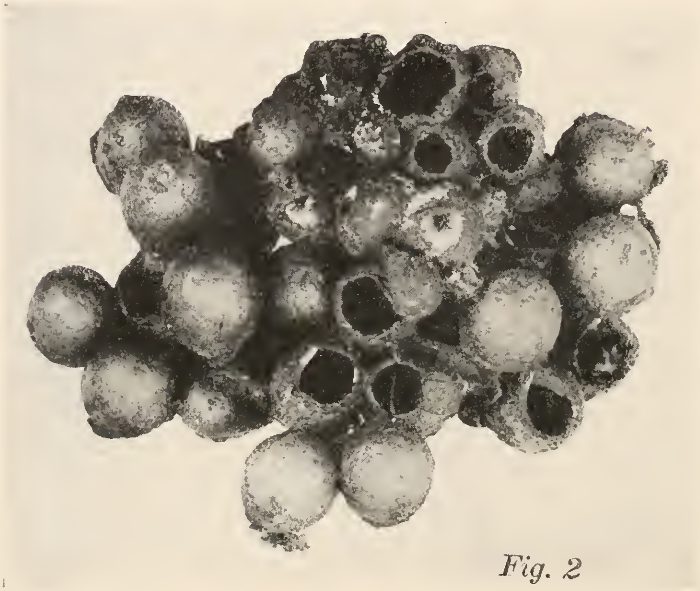

Fig. 2. Comb of *Bremus affinis* showing exposed larvæ. Natural size.

In this connection the writer would like to call attention to the fact that some of our American *Amarsipœa*, *e.g.*, *Bremus affinis*, *occidentalis*, and *terricola*, have the same habit (cf. Fig. 2). Quite contrary to this treatment of the larvæ is that of the other American and European *Amarsipœa* whose habits have been studied, for the latter carefully keep their larvæ covered with wax (cf. Fig. 3).

Because of this difference in habit, the present writer proposes the name *Phaneroschadonenta* (from Greek *phaneros*, visible, and *schadon*, a bee larva) for those *Amarsipœa* whose larvæ are visible during the greater part of their development, and the name *Cryptoschadonenta* (from Greek *cryptos*, hidden) for those *Amarsipœa* which keep their larvæ covered with wax.

In addition to the very loosely connected cocoons and the uncovered larvæ, the *Phaneroschadonenta* exhibit a number of other peculiarities which mark them as a distinct group. Among these may be mentioned (1) the short antennæ of the males, (2) the similarity in form between males and workers, and (3) the extreme shortness of the head.[6] This last character is coupled with a short tongue, which makes it necessary for this group of bumblebees to perforate long-tubed flowers in order to secure the nectar. Because of this habit they have been given the name *Disteleologists* (cf. Haeckel, '66; Müller, '79; and von Buttel-Reepen, '14), a subject which the writer intends to discuss in another paper.

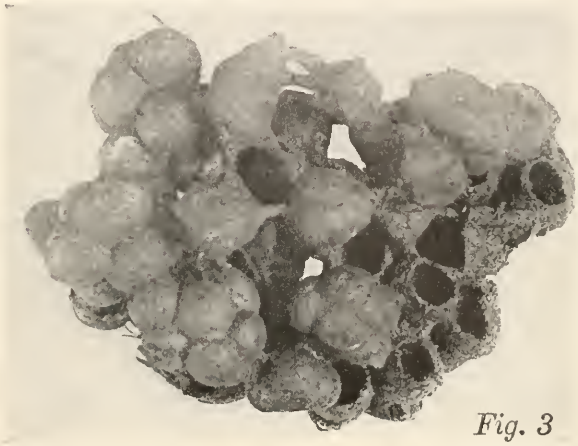

FIG. 3. Comb of *Bremus impatiens*, a typical example of the *Cryptoschadonenta*. Natural size.

The *Phaneroschadonenta* also have the following interesting characteristics: (1) they construct—usually near the center of the comb—one or more bulky, waxen tubes in which they store large quantities of pollen (cf. Figs. 4 and 5); (2) the wax which they produce is unusually dark and brittle, as if mixed with black soil; and (3) their males, like those of the genus *Psithyrus*, are rather sluggish as compared with those of other species.[7] The writer would here also like to point out that the *Phaneroschadonenta* appear early in the spring, that they do not nest on

[6] Cf. Radowszkowski ('84), Friese and von Wagner ('10), Vogt ('11), Franklin ('12/'13), and Krüger ('20).

[7] Cf. Schmiedeknecht ('78), Hoffer ('82, '82/'83), Saunders ('09), Sladen ('12), and Frison ('17).

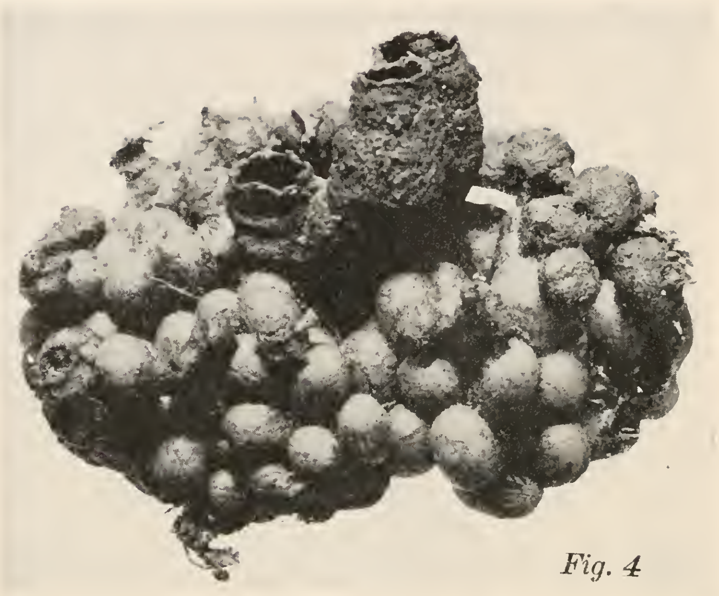

FIG. 4. Comb of *Bremus affinis* showing three pollen cylinders. Natural size

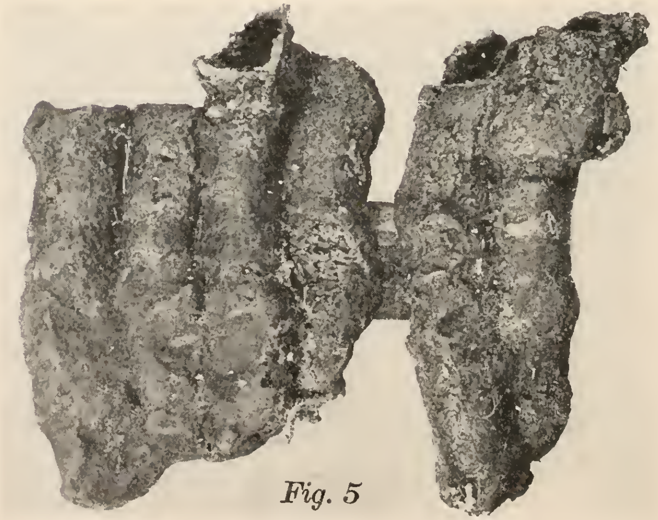

FIG. 5. Six pollen cylinders taken from a nest of *Bremus affinis*. Natural siz
Sometimes more than a dozen of these pollen cylinders are found in one nest.

the surface of the ground, and that—as far as known—they do not occur in South America.

However, it may be necessary to use the terms *Phanero-* and *Cryptoschadonenta* only provisionally, since Sladen ('12, pp. 36–37 and 185) states that *Bremus latreillellus*, a *pocket-maker* (*Marsipœa*), also leaves its larvæ uncovered. This seems strange, since the other *Marsipœa* studied are very solicitous to keep their larvæ completely covered with wax (cf. Fig. 6), and this fact suggests the possibility that Sladen's ('12) observations on *Bremus latreillellus* may have been made during extremely hot weather, when, due to the softening of the wax, all bumblebee larvæ are likely to become exposed. Should subsequent observations confirm Sladen's ('12, pp. 37 and 185) statement concerning *Bremus latreillellus*, other terms will have to be substituted for *Phanero-* and *Cryptoschadonenta* to bring out the distinctness of this group of bumblebees.

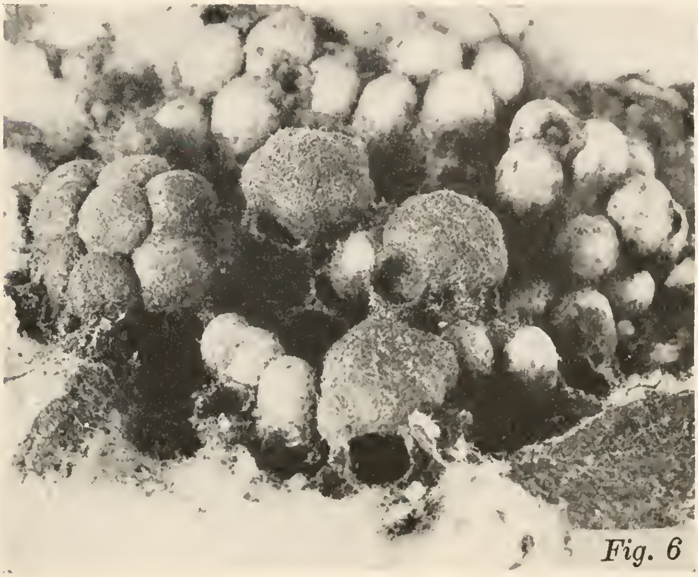

FIG. 6. Comb of *Bremus americanorum* showing (*a*) three pockets, and (*b*) worker larvæ completely enclosed by wax. Natural size.

The various American and European species of bumblebees whose methods of rearing their young have been studied up to the present are listed in Table I. with the suggested modifications of Sladen's ('99 and '12) scheme.

TABLE I.

AMARSIPŒA.

Phaneroschadonenta.

1.	*Bremus affinis*	American Species.
2.	" *occidentalis*	
3.	" *terricola*	
4.	" *lucorum*[8]	European Species
5.	" *terrestris*[8]	

Cryptoschadonen

6.	*Bremus bimaculatus*	American Species.
7.	" *impatiens*	
8.	" *perplexus*	
9.	" *ternarius*	
10.	" *vagans*	
11.	" *auricomus*[9]	
12.	" *separatus*	
13.	" *jonellus*[8]	European Species.
14.	" *lapidarius*[8]	
15.	" *lapponicus*[8]	
16.	" *pratorum*[8]	

MARSIPŒA.

17.	*Bremus americanorum*	American Species.
18.	" *fervidus*	
19.	" *hortorum*[8]	European Species.
20.	" *ruderatus*[8]	
21.	" *distinguendus*[8]	
22.	" *latreillellus*[8]	
23.	" *agrorum*[8]	
24.	" *derhamellus*[8]	
25.	" *helferanus*[8]	
26.	" *muscorum*[8]	
27.	" *sylvarum*[8]	

Concerning the relative temper of the *Marsi-* and *Amarsipœa*, Sladen ('99, pp. 230–231) says: "It will be noticed that the *pouch-makers* include roughly the timid species. The *pollen-storers*, on the contrary, consist of the bolder species." This is not true of our New England *Marsipœa*, *e.g.*, *Bremus americanorum* and *Bremus fervidus*, these being the most vicious bumblebee species with which the present writer has come in contact, while most of our *Amarsipœa* are comparatively gentle.

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[8] According to Sladen ('12, pp. 152–153).

[9] According to Frison ('17, pp. 284).

It will be interesting to see whether the various bumblebee groups which have been established on the basis of structure are really homogeneous species, when their habits are studied from a taxonomic viewpoint. In the light of our present knowledge, we should expect the seven American groups (cf. Franklin, '12/'13) of the genus *Bremus* to belong to the biological subdivisions as indicated in Table II.

TABLE II.

AMARSIPŒA.

Phaneroschadonenta.

1. *Terrestris* group.

Cryptoschadonenta.

2. *Auricomus* group.[10]
3. *Fraternus* group.[10]
4. *Kirbyellus* group.[11]
5. *Pratorum* group.

MARSIPŒA.

6. *Borealis* group. [11]
7. *Dumoucheli* group.

If Krüger's ('16 and '20) subdivision of the Bremidæ into two sections is really as far-reaching as it appears to be, we should expect the members of the *Borealis* group to belong to the *Marsipœa*, and those of the *Kirbyellus* group to the *Amarsipœa*, since the spine is present in the former, but absent in the latter.

It will also be interesting to investigate the methods which the Bremidæ employ in feeding the larvæ of the genus *Psithyrus*, since certain species of the latter, *e.g.*, *Psithyrus variabilis*, breed in the nests of the *Marsipœa*, while others, *e.g.*, *Psithyrus ashtoni*, are parasitic on the *Amarsipœa*.

Due to a lack of sufficient material, the writer has been unable to determine whether the species for which Robertson ('03) erected the genus *Bombias* have any clearly defined biological peculiarities [12] which would serve as suitable biological characters for distinguishing this group.

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[10] Belonging to the genus *Bombias* of Robertson ('03).

[11] Probably.

[12] The males of this group, like those of closely related European species, have their own peculiar habits (cf. Schmiedeknecht, '78; Hoffer, '82/'83; Robertson,

Summary.

1. Contrary to the assertions of Wagner ('06), biological characters are of considerable importance in determining the relationships between the various species of bumblebees, while structural characters frequently make it possible to draw inferences as to the habits of these insects.

2. Our North American *Marsipœa*—at least *Bremus americanorum* and *Bremus fervidus*—feed only their worker brood through pockets, while those larvæ which are destined to become queens and males are fed by regurgitation.

3. The use of the terms *Pollen-primers*, *Carder-bees*, and *Long-faced humble-bees* (cf. Sladen '12) is impracticable in any comprehensive classification of the Bremidæ.

4. Von Buttel-Reepen's ('03) explanation of the pollen-priming habit of bumblebees is not substantiated by the biology of some of our North American species.

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BIOLOGICAL BULLETIN

OBSERVATIONS ON THE LIFE-HISTORY OF *AMŒBA PROTEUS*.[1]

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There is still considerable diversity of opinion concerning the life-history of *Amœba proteus*. Some hold that it reproduces exclusively by binary fission; others do not agree with this.

Carter ('56) asserts that he observed the nucleus in a given specimen, break up into several small nuclei each of which associated itself with a bit of cytoplasm; and that he then saw the membrane of the amœba break and the small nuclei with their surrounding cytoplasm escape into the external medium and move away by amœboid movement.

Wallich ('63), Scheel ('99), Calkins ('05 and '07), Metcalf ('10), Hausman ('20), and Taylor ('24) maintain that they made similar observations. Scheel and Calkins contend that the amœbæ encyst before the nucleus breaks up and Calkins holds that there is a multinucleated generation which ends by sexual activity. Calkins says, "The fertilized cell of Amœba (unknown at present) gives rise to a young amœboid organism formerly known as *Amœba proteus*." Later ('07) he sectioned the amœbæ on which these observations were made and maintains that he found a process of internal fertilization very similar to endomyxis.

Metcalf ('10) asserts that there are two methods of reproduction in which fragmentation occurs; one in which the parent amœba breaks up liberating minute amœboid forms which develop into large amœbæ; another in which small amœboid forms are liberated from the parent by a gemmule formation. These amœboid forms he asserts develop flagella, then fuse in pairs, after which the zygotes thus formed develop into large amœbæ.

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[1] These observations were made while the authors were working under Dr. S. O. Mast, to whom they are very grateful for timely criticism and helpful suggestions.

Taylor gives a detailed description of the nuclear processes accompanying fragmentation. She maintains that the small fragments as they emerge from the parent amœba are cysts which hatch out after varying lengths of time.

All of these observations indicate that *Amœba proteus* at times fragments, forming numerous small amœboid forms, and they appear to indicate that these small forms develop into the large forms which are usually studied. Hausman contends that he actually observed the transformation. There are, however, some prominent investigators, who hold that the evidence presented is not conclusive; for example, Schaeffer, ('26, p. 111.) who says, "We find no proof that the life cycle of the common large ameba includes more than . . . reproduction by fission." The following observations have a definite bearing on this question:

During the last two years we have had under close observation numerous cultures of *Amœba proteus*, started from a few specimens collected in the summer of 1924. During this time it was repeatedly noticed in various cultures that the amœbæ multiplied very rapidly for a while, then suddenly practically disappeared. At first these cultures were all discarded, except a few which were saved for the other protozoa living in them. These were set aside and observed from time to time. After about a week, large numbers of minute amœbæ were found in some of them, and several weeks later numerous large amœbæ.

In further observations it was discovered in several cultures of large amœbæ, (1) that after a period of rapid multiplication by fission the specimens became increasingly more sluggish, darker and more granular in appearance; (2) that they began to decrease in number; and (3) that as the large amœbæ decreased small ones appeared and increased. This did not occur in all cultures, for in some all amœbæ died.

This disappearance of the large amœbæ was observed to occur in several cultures as the solution changed from acid to alkaline in reaction (pH 6.8 to 7.4) or *vice versa*. In other cultures, however, there was no such change, in these the solution remained almost constant as to bacterial content, clearness, amount of food and hydrogen-ion concentration.

Similar results were obtained in cultures on hollow ground slides. All of these cultures consisted of a few drops of filtered culture fluid. One large amœba was put into each of about half of them, and none in the rest. Numerous small amœboid forms appeared in many of the former but in none of the latter. A description of a typical experiment follows:

A large sluggish individual was selected, washed three times in about 5 cc. of distilled water and placed on a hollow ground slide in culture fluid which had been passed through number 50 filter paper. This solution contained no amœbæ that could be seen under a highpower dissecting binocular, or a 1.9 mm. water-immersion objective. The amœba was observed from time to time. The second day after it had been put into the culture fluid on the slide, it became extremely sluggish and remained so. The following morning it had disappeared and in the region where it had been, there were from 150 to 200 minute amœboid forms, about 10 μ in length. A drop of fresh sterile hay infusion was now added every other day for about a week and it could clearly be seen that the small amœboid forms were becoming distinctly larger. Unfortunately, at the end of this time too much solution was added and the forms died.

Following these experiments several amœbæ were isolated and closely watched for a long period of time. The process of fragmentation was actually observed to occur in a number of cases.

The results obtained seem to prove conclusively that *Amœba proteus* at times breaks up into small amœboid forms but they do not prove that these small forms develop into large ones. The evidence presented in the following paragraphs appears, however, to prove this.

On February 19, 1925, two grams of timothy hay were added to 1000 cc. of spring water and boiled for ten minutes. While still hot some of the fluid was poured into a sterile 100 cc. pyrex flask. This was then plugged tightly with cotton. When the flask had cooled a few drops of old culture fluid, which had been passed through number 50 filter paper, were added. The flask was then again plugged with cotton and allowed to stand for a week. Then this culture, free from amœbæ as shown by careful observation, was inoculated by one amœba which had been washed in several

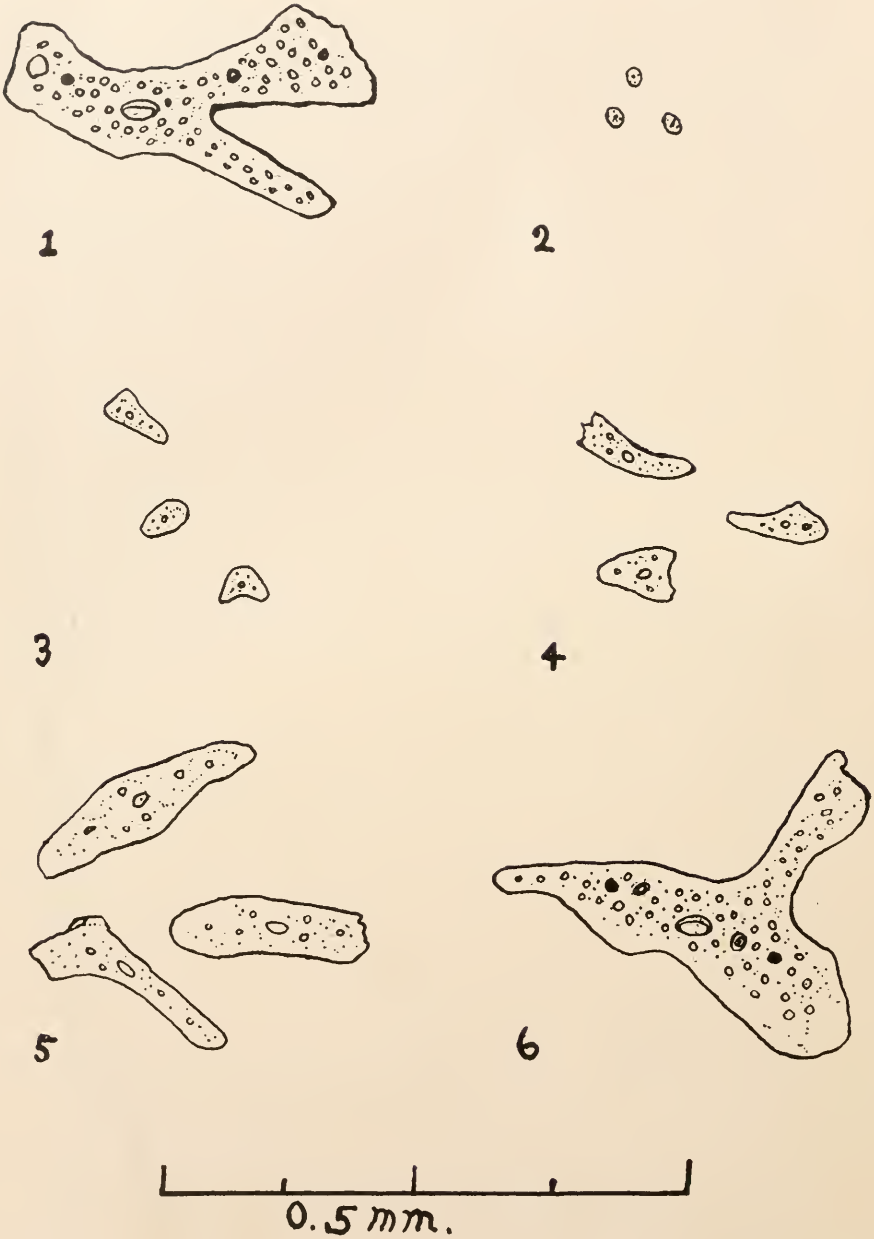

Fig. 1. Camera lucida sketches of some of the largest amœbæ in a culture at different time intervals, showing growth of small amœbæ into large amœbæ. 1, one of the original amœbæ which fragmented, giving rise to small amœbæ; 2,

changes of distilled water. After this there was added each day for food about 5 cc. of fresh sterile culture fluid like the original culture fluid. All pipettes used in handling the amœba and the culture fluid were repeatedly sterilized in order to eliminate contamination.

The culture was poured into a sterile shallow dish and thoroughly examined every few days. It was found that the individual multiplied by fission until in about two weeks there were perhaps fifty large amœbæ in the culture. Then fission appeared to cease and the amœbæ assumed a dark granular color, after which they began rapidly to disappear so that by the end of the third week there were no large amœbæ present. If there had been one large amœba left it could not have escaped the rigid inspection to which the culture was subjected. The culture was now set aside for a week, after which it was again examined. It now contained numerous small amœbæ the length of which varied from 10 to 40 μ, but no large amœbæ. For over a month following this the culture was repeatedly closely observed. The amœbæ increased in size until by the last of April there were numerous specimens which measured 300–600 μ in length.

On September 17, 1926, fourteen cultures made up in a modified Ringer's solution were inoculated with amœbæ from an ordinary spring water culture after being washed in six changes of the Ringer's solution. At the end of four days all of the large amœbæ had disappeared and soon after this small amœboid forms were observed in great numbers. From this time on the cultures were thoroughly examined at the end of each week and after each examination camera lucida sketches were made of the largest amœbæ found in each culture. Some of these sketches are herewith reproduced (Fig. 1). They show that the amœboid forms increased from an approximate average volume of 400 cubic micra to one of 250,000 cubic micra and that this increase required two months. During this time, however, it was observed that these small amœbæ occasionally divided (Fig. 2). All of

small amœbæ produced by fragmentation of original large amœbæ; 3, largest amœbæ in culture ten days after fragmentation; 4, largest amœbæ in culture four weeks after fragmentation; 5, largest amœbæ in culture six weeks after fragmentation; 6, largest amœbæ in culture eight weeks after fragmentation.

these cultures and the one mentioned in the preceding paragraph, were thoroughly examined at least twenty-five times during the two months growth period, and no large amœbæ could be discovered until the end of two months as stated. It is, therefore, evident that even one large amœba could not have remained in the large form unobserved during the growth period of nearly two months.

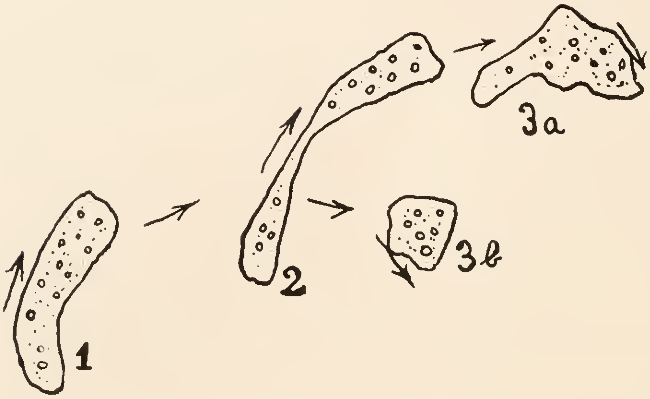

FIG. 2. Camera lucida drawings of a small amœba just before division (1) and (2) and immediately after division (3*a*) and (3*b*).

It is evident that the small forms develop into large ones and that as they do so they divide from time to time.

SUMMARY.

Individuals of *Amœba proteus* sometimes break up into from 100 to 300 amœboid forms. These amœboid forms gradually become larger until at the end of about two months they are as large as the original specimens. During the increase in size division occurs from time to time.

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AN ANALYSIS OF THE SPAWNING HABITS AND SPAWNING STIMULI OF *CUMINGIA TELLINOIDES*.[1]

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Since the discovery of a slight lunar periodicity in the spawning of *Chætopleura apiculata*, the writer has studied the spawning seasons of a considerable number of animals in the hope of finding other more clearly marked cases. The conclusion of this study is that the phenomenon is of rare occurrence at Woods Hole. *Cumingia tellinoides* is the one species studied critically to date that shows a lunar periodicity. Three other species whose study has been completed show absolutely no periodicity. Others being studied require further investigation before they can be passed upon with any degree of certainty. If semi-scientific reports are trustworthy, this type of periodicity is much more common in the tropics, since several fairly well authenticated cases are reported from the Tortugas and elsewhere.

A part of the data presented in this paper was read before the American Society of Zoölogists at their annual meeting in December, 1922. Since that time the study has been continued and the results are finally presented for publication in their completed form.

SECTION I.

THE SPAWNING SEASON.

The Duration and Characteristics of the Spawning Season.

Cumingia begins to spawn about the middle of June and continues with variations of activity until the middle of September and sometimes until the first of October. During 1926 the first eggs were obtained on June 21 and the last ones on September 21. The heaviest spawning occurs during the last week in June, July and the first half of August, as stated by Morgan (*Jour. Exp. Zoöl.*, 1910). The production of eggs by each female is continuous and covers the entire period. If *Cumingia* which have spawned vigorously in the laboratory are returned to their normal habitat for three weeks or a month, they will again set free large quantities of eggs or sperm showing that the supply is replenished.

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[1] From the Osborn Zoölogical Laboratory, Yale University, New Haven, Conn., and the Marine Biological Laboratory, Woods Hole, Mass.

Experiment No. 1.—July 7, 1924. A stake was driven off Ram Island and eighteen *Cumingia* were planted at its base. All these had just spawned in abundance. On August 7, seven of these *Cumingia* were again brought to the laboratory and treated as usual to induce spawning. After forty minutes two females had spawned heavily and three males had shed sperm abundantly. One other female spawned after one and one half hours. One of the seven did not spawn. The eggs of all these were fertilized and grew into normal veligers.

Experiment No. 2.—July 6, 1926. Eighteen *Cumingia* that had just spawned heavily were planted off Ram Island. On August 1, nine of these were again brought to the laboratory. Three females spawned actively, three males shed sperm in quantity, three did not spawn.

These two experiments indicate that *Cumingia* spawns more than once in a season and that the production of eggs and sperm is continuous.

There are indications that each female spawns two or three times during the season when left in the natural habitat. This inference is founded upon the facts that the eggs are constantly produced and that spawning seems to be most common at full moon and the days following full moon. Since there is no direct evidence on this point, I do not care to insist upon it, except to refer to the schedule of spawning experiments. (See tables, Section III. on lunar periodicity.) There are clearly marked variations in spawning activity which appear to be associated with phases of the lunar cycle.

Orton 15 has advocated the theory that the duration of the spawning seasons of animals is determined by temperature. According to his conception, spawning by any summer breeding species begins in the spring when the water reaches a certain temperature and ends in the fall when the water drops below that temperature. It may be in order to say that this theory is true only in a very general sense, since the species that have been studied by me do not agree among themselves in this regard. Thus *Cumingia* began to spawn on June 21, 1926, when the temperature of the water was 60° F. and quit about September 25 when the temperature was 65° F. *Bugula flabellat* began spawning on June 20 in 1926 when the temperature was 60° F. and continued until November 26, when the temperature was 47° F. *Bugula* usually begins to shed its larvæ by June 10 and continues until late in November, which makes the discrepancy in temperature less pronounced, June 10 temperature being approximately 55° F., November 25 temperature 45° F. *Chætopleura* begins to spawn about June 25, temperature 60° to 65° F. and ends early in September, temperature 65° to 70° F.

Spawning is caused at particular times in nature by various specific stimuli and it is not determined by temperature alone.

Orton gives convincing evidence to show that temperature is the chief factor in determining the limits of the spawning seasons of marine invertebrates. He, however, dismisses the factor light with too little emphasis. Although most tropical animals may spawn throughout the long summer in accordance with his theory, the Palolo worm spawns on one or two days only; and the ripening of its gametes is influenced by light according to several investigators. Light plays a part both in the maturing of the gametes and in spawning in several well-known cases. A very striking example is *Dictyota* at Beaufort, N. C., as described by Hoyt. Temperature, no doubt, is the chief factor in determining the spawning season just as it is also the most important factor in growth and all protoplasmic activity.

SECTION II.

Artificial Spawning Stimuli.

I. Shock.

As stated in my paper on *Chætopleura* (Biol. Bull., 1922), certain marine invertebrate animals will spawn when placed in an artificial or unusual environment, stimulated apparently by shock. As a case in point, if the tube forming annelid Hydroides is removed from its shell and the exposed worm placed in a dish of sea water it will spawn at once, the eggs or sperm seeming to come from every nephridium of the body. This worm will always spawn under these circumstances whether the gametes are fully mature or not. Half-grown eggs are as readily spawned as mature ones. Hydroides will spawn in the spring before any of the eggs are even approximately mature.

In like manner, *Cumingia* spawn readily in the laboratory. It is only necessary to place them in a bowl of sea water and allow them to remain undisturbed for half an hour or forty-five minutes. They usually begin to spawn in half an hour. The gametes are expelled through the dorsal siphon which is extended to a great length. As the eggs or sperm accumulate in the siphon they are thrown out forcibly by sudden whip-like contractions of these organs. During the height of the breeding season it frequently happens that every individual brought to the laboratory spawns. It is not to be supposed that all would have spawned on that particular day if left in their natural environment. There is then something about the treatment which involves digging them from the sand and subjecting them to various unusual disturbances which stimulates spawning. It is safe to conclude that this spawning stimulus is shock as in the case of Hydroides. Spawning takes place just the same when sea water is allowed to flow gently through the dish so that the accumulation of CO_2 is not a factor. It is to be noted that shock is an abnormal stimulus. It may be said again that laboratory experiments are frequently unreliable guides to natural behavior and may lead to misinterpretation if depended upon too implicitly.

Chemical Stimuli.

The question next arises: do the sexes stimulate each other in any way? The question as to whether the females are stimulated to spawn by the presence of the male and vice versa has frequently been discussed in connection with various species. Some very clear cases of the existence of such a chemical stimulus have been discovered. This, for instance, is the case in *Nereis limbata* as described by Lillie and Just. When placed together in a dish of sea water both sexes are excited at once to remarkable activity, and while swimming excitedly they expel their sexual products with violent contractions. Galtsoff has recently shown that the female oyster will spawn if living oyster sperm is added to the water. This is opposed to the published statements by Nelson who has thought that the oyster spawns on particularly warm days and that general spawning on oyster beds is induced by temperature. I am under the impression that he is correct in this statement but the implied conclusion that temperature is the only spawning stimulus may be misleading. He shows, however, that spawning occurs on the peaks of rising temperatures which indicates that changes in temperature upward cause males to shed and hence general spawning by all nearby oysters results. Spawning by oysters may take place at any temperature between 68° and 85° F. (Churchill and Nelson).

It was found in the case of *Cumingia* that there is no perceptible stimulus from the opposite sex. They appear to spawn quite as readily when isolated as when in the same dish. Experience has shown that the best way to obtain clean eggs in convenient form for study is to wash the animals free from debris and isolate them in small stender dishes half filled with sea water, or enough water to cover the animal. Both males and females will shed their gametes when so isolated. The eggs are thus obtained free from sperm and may be artificially fertilized at will. Drew was the first to use this method and he was under the impression that drying accentuates or constitutes the spawning stimulus. Morgan in 1910 noted that *Cumingia* will spawn when isolated (*Jour. Exp. Zoöl.*, Vol. 9, p. 595). He thinks, however, that the presence of spermatozoa in the water may incite the females to spawn more promptly than they otherwise would. The stimulus in this case, if authenic, might be either chemical or physical. One is likely to gain the same impression by watching the spawning of *Chætopleura* (*Chiton*) because when males and females are placed together in a dish of sea water, the males always shed their products first, and are followed promptly by the females. So far as could be learned, however, spawning by the females occurred as promptly without spermatozoa as with them.

It is the writer's belief that the spermatozoa have no effect. The mechanical shock of removing the animals from their normal situations furnishes all the stimulus that is required to induce spawning. There is no perceptible stimulus from the opposite sex either chemical or physical. This is shown by a series of experiments designed to test the theory. The three described herewith are representative. The method was to collect *Cumingia* in large numbers. Half of them were isolated in small stender dishes and covered with sea water in the usual way. The other half were treated in the same way except that they were all put in a large crystallization dish so that they might receive chemical stimuli from each other if such exist. The time of spawning is shown in the two columns of the tables and they may be readily compared.

Experiment No. I.

August 21. Collected 20 *Cumingia*. Ten were isolated in stender dishes and ten were placed in a common dish. Experiment set at 12:30 P.M.

10 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	10 in Common Dish Spawned as Follows.
1:15 P.M. one female.............	45 minutes	
1:16 P.M. one male..............	46 "	
1:20 P.M. one male..............	50 "	
1:23 P.M. one male..............	53 "	
	55 "	One male shed 1:25 P.M.
	57 "	One male " 1:27 P.M.
1:29 P.M. one female.............	59 "	
	60 "	One male " 1:30 P.M.
	61 "	One female " 1:31 P.M.
	67 "	One male " 1:37 P.M.
	68 "	One male " 1:38 P.M.
	70 "	One male " 1:40 P.M.
	71 "	One female " 1:41 P.M.
1:55 P.M. one female.............	85 "	
2:05 P.M. one female.............	95 "	

Comment: Three of the isolated individuals and two in the common dish failed to spawn. Comparison shows that those in the common dish did not spawn more promptly than the isolated individuals. It is noteworthy that those in the common dish spawned close together in point of time. This was also true in Experiment No. 3, but not in No. 2.

Experiment No. 2.

August 27. Collected 34 *Cumingia*. Isolated seventeen in stender dishes and placed seventeen in a common dish. Experiment set at 5:55 P.M.

17 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	17 in Common Dish Spawned as Follows.
	30 minutes	One female at 6:25 P.M.
One male at 6:27 P.M.	32 "	
One male at 6:28 P.M.	33 "	
One male at 6:29 P.M.	34 "	
	35 "	Two males at 6:30 P.M.
	36 "	One male at 6:31 P.M.
One female at 6:32 P.M.	37 "	
	38 "	One male at 6:33 P.M.
	39 "	One male at 6:34 P.M.
	41 "	One female at 6:36 P.M.
One male at 6:37 P.M.	42 "	One male at 6:37 P.M.
One female at 6:37 P.M.	42 "	
One male at 6:39 P.M.	44 "	
	47 "	One male at 6:42 P.M.
	48 "	One male at 6:43 P.M.
One male at 6:44 P.M.	49 "	
One female at 6:45 P.M.	50 "	
Two males at 6:46 P.M.	51 "	One female at 6:46 P.M.
	52 "	One female at 6:47 P.M.
	55 "	One male at 6:50 P.M.
One female at 6:51 P.M.	56 "	
One female at 6:54 P.M.	59 "	

Comment: Five isolated individuals did not spawn within the hour and five of those in the common dish did not spawn. There is no evidence that they receive a chemical stimulus from each other calculated to induce spawning. They spawn as readily when isolated as when in the same water.

Experiment No. 3.

August 28. Collected 24 *Cumingia*. Isolated twelve in stender dishes and placed twelve in a common dish. Experiment set at 1:30 P.M.

12 Isolated Individuals Spawned as Follows.	Time Elapsed before Spawning.	12 Individuals in Common Dish Spawned as Follows.
	2 minutes	One male at 1:32 P.M.
1:48 P.M. one male	18 "	
	25 "	One male at 1:55 P.M.
	27 "	One male at 1:57 P.M.
1:58 P.M. one male	28 "	One male at 1:58 P.M.
1:59 P.M. one male	29 "	One male at 1:59 P.M.
	30 "	One female at 2:00 P.M.
	35 "	One male at 2:05 P.M.
	38 "	One male at 2:08 P.M.
	39 "	One male at 2:09 P.M.
2:10 P.M. one female	40 "	
2:13 P.M. one female	43 "	
2:15 P.M. one male	45 "	
2:16 P.M. one male	46 "	
2:17 P.M. one female	47 "	
2:20 P.M. one female	50 "	
2:22 P.M. one female	52 "	

Comment: Two of the isolated individuals and three in the common dish did not spawn within an hour. As a matter of chance most of the females got into the isolated dishes.

SECTION III.

NATURAL SPAWNING STIMULI.

I. Lunar Periodicity.

If *Cumingia* spawns most frequently at the period of the full moon, it is evident that there is some cosmic stimulus which varies with the moon's phases to which spawning is due. The two chief variable quantities are light, and tides, or pressure. There is at present no scientific explanation of lunar periodicity although numerous cases are known.

It has been the writer's chief interest during the past five years to learn whether the phenomenon is rare or of common occurrence among animals at Woods Hole. The data bearing on this subject are given in a short forthcoming paper, but the findings in reference to *Cumingia* are given here in greater detail.

A careful study and comparison of data collected during the past five years shows that there is a lunar periodicity in spawning, although it is not as well marked as in *Nereis limbata* or the Suez sea urchin studied by Fox.

There is no clock-like regularity in the spawning of *Cumingia* in nature. Not all individuals spawn promptly at the full moon. Spawning in fact covers a period of two or three weeks in each month. We know this by the quantities of eggs spawned in the laboratory by any particular lot of sexually mature adults brought in. Some set free a maximum quantity, some a small quantity and some only a few eggs or none at all. The last are considered to have spawned recently. By this indirect reasoning, one learns that there is no one particular day on which general spawning occurs. It is likely that spawning occurs when the gonads and their ducts become filled to capacity and this internal pressure no doubt constitutes a second natural spawning stimulus. If the production of gametes were continuous, and uniform in rate, spawning might occur on any day in the month except for this cosmic stimulus which brings about a more or less marked rhythm. I am of the opinion that there is a rhythm in the rate of production of the gametes as well as in the spawning of them. In any case, the only time in the month during which most of the gonads seem empty and during which spawning stimuli seem to be suppressed is the period of the first quarter. Beginning at full moon the heavy spawning is sometimes completed before new moon but more frequently not until near first quarter. In many respects *Cumingia* resembles *Toxopneustes*, the Beaufort sea urchin, whose periodicity was described by Tennent. The production of gametes in this species is rapid and the gonads are soon replenished after spawning occurs.

It should be noted here that there is a distinction between stimuli which bring about the act of spawning and those which bring about the maturing of the gametes. The latter may be an evenly continuous process or it may be enhanced at certain times. The former is merely the act of extruding these gametes and may be induced by some external stimulus, such as those due to the moon in its various phases, or to any other external stimulus.

In the following paragraphs I give the history of this study of *Cumingia* as constituting an important part of the experimental basis for the conclusions drawn.

During the greater part of the breeding season of *Cumingia*, no lunar periodicity in spawning is likely to be observed unless attention is directed to it. *Cumingia* has been used as a source of embryological material for class work for many years and only rarely has difficulty been experienced in obtaining eggs in abundance at any time that they have been needed. It appeared that eggs could be obtained at any time during the summer. It was only after receiving a suggestion from Heilbrunn that any convincing evidence of periodic spawning was obtained. He said that *Cumingia* would spawn a second time after its season had apparently ended in August. Morgan gives the spawning season of *Cumingia* as June, July, and August, so that he evidently overlooked the September spawning after the break in August.

It was at Heilbrunn's suggestion that I undertook experiments late in the summer of 1922, when, as he said, a break could be expected.

During the week of August 23 to September 1, 1922, no eggs could be obtained from *Cumingia*, although the characteristic spawning reactions were carried out as usual. The siphons were extended to great length and the whip-like lashing of these organs was carried out. All that was expelled, however, was a considerable amount of mucus containing at best a few immature or defective eggs. There were no mature eggs in the ovaries.

Bean, working in the same laboratory, was likewise unable to obtain eggs during this period for experimental purposes. Bean worked constantly with *Cumingia* during the summer of 1922 and he thought that he detected a periodicity during the height of the breeding season. The maximum spawning periods, according to his statement, occur at new and full moon, being therefore bimonthly. This is interesting, if correct, as indicating that the spawning stimulus might be associated with the tides rather than with moonlight. Further study during the summers of 1923, 1924, 1925 and 1926 shows conclusively that the maximum spawning comes at full moon and is not bimonthly.

The table for 1922 gives the exact data as obtained by the writer. It will be seen that spawning activity is revived somewhat before full moon after a period of complete cessation. Furthermore a careful study of the tables for the years 1922, 1924 and 1926 shows that the heaviest spawning occurs at the period of the full moon until new moon, and that the period of the first quarter is the period of restricted spawning. This behavior of *Cumingia* can scarcely be explained on any other ground than as a lunar effect.

There is no adequate way of describing the variations in spawning that are so obvious to the experimenter. However, the tables of data appended to this section, together with the comments written at the time the experiments were performed, must suffice to explain the basis of the conclusions arrived at. Although they are long, there seems to be no way to give the evidence more briefly. I give the data for three years only as representative.

Discussion.

There has been much speculation about the cause of lunar periodicity in spawning, but it has for the most part remained speculation and guessing. It can never be solved except by experiments similar to those devised by Mayer. Neither *Cumingia* nor *Chætopleura* is suitable for a study of the causes which have produced lunar periodicity. In the first place, periodicity is not clear-cut in these species and in the second place they can be observed only under laboratory conditions which have been shown to be unreliable. They are affected by shock whereas some species are apparently not so affected.

A casual survey of known cases of lunar periodicity shows a general similarity in all and it is likely that the underlying causes are the same in all. A study which undertakes to explain these phenomena should include a search for other cases and especially all worms that are known to show a lunar periodicity should be studied in detail for purposes of comparison. However, speculation and comparison can scarcely explain it. There is need for experimental methods and the Palolo worm, and the Suez sea urchin or possibly *Nereis limbata* are among the most favorable species for study. It should be a comparatively simple problem to subject them to artificial light, especially polarized light, in addition to all the light to which they are accustomed, to see if they can be thrown out of tune. The Palolo worm seems to offer the best opportunity because of its definite and predictable time of spawning, so that any change from this time could be regarded as an experimental modification.

Summary.

1. The spawning season of *Cumingia* extends from the middle of June until the middle or end of September. The most active spawning usually includes the last week in June and ends about August 20. Each female spawns more than once and the production of gametes is practically continuous.

2. The spawning is heaviest from full moon to new moon and least at first quarter thus showing a lunar periodicity.

3. *Cumingia* is greatly affected by shock and rough treatment so that sexually mature individuals spawn promptly when brought to the laboratory, after being placed in a bowl of quiet sea water.

4. There are apparently no chemical stimuli by which the sexes stimulate each other to sexual activity as is the case in *Nereis limbata* and the oyster. They spawn as readily when isolated as when in close proximity.

5. It is shown that temperature is not the only factor which determines the duration of the spawning season and periods of spawning.

SPAWNING EXPERIMENTS. 1922.

Cumingia tellinoides.

Table No. I.

Cumingia spawned vigorously during the first half of August but stopped suddenly about August 15. Several investigators were experimenting upon Cumingia eggs at that time and noted this fact. All collections were made at low tide or within an hour of low tide at the convenience of the supply department of the laboratory.

* *September 21, new moon.*

I. 8/23. No eggs spawned. 1 male shed sperm abundantly. 17 did not spawn.

II. 8/24. No eggs and no sperm.

III. 8/27. No eggs spawned. 4 males shed sperm small amount. 14 did not spawn.

* *September 28, first quarter.*

IV. 9/1. 5 females spawned in quantity. 6 males shed sperm, 3 in quantity, 3 in small amount. 7 did not spawn.

Comment.—This revival of spawning after the spawning season had apparently ended is surprising. The quantity of eggs shed is equal to mid-season spawning.

V. 9/2. 4 females spawned in quantity, 2 maximum, 2 one half maximum. 5 males shed sperm, 3 heavy and 2 light. 9 did not spawn.

* *September 5, full moon.*

VI. 9/5. 6 females shed eggs, 4 of them maximum. 5 males shed sperm, 3 abundantly and 2 light. 4 did not spawn.

VII. 9/6. 6 females spawned, 4 maximum, 2 one half maximum. 5 males shed sperm, none maximum, mostly light. 8 did not spawn.

Note.—This cessation or reduction of spawning in August is typical as shown by five years' experience. Spawning is also regularly revived in September. It is most marked when full moon falls during the first week of the month; and less marked when it is near the middle of the month. This record for 1922 led the writer to study these phenomena in detail in succeeding years. They can scarcely be explained on any other ground than a lunar cycle effect.

SPAWNING EXPERIMENTS. 1924.

CUMINGIA TELLINOIDES.

Table No. II.

* *July 2, new moon.*

I. 7/5. 6 females spawned well (maximum). 5 males shed well (maximum). One did not spawn.

II. 7/8. 4 females spawned well (maximum). 5 males shed well (maximum). 3 did not spawn.

* *July 9, first quarter.*

III. 7/9. 100 per cent. spawned. 3 females spawned, 2 heavy, one light. 4 males shed, 2 heavy, 2 light.

Remark.—Heavy spawning activity during the past week and nearly 90 per cent. of the individuals spawning approximately maximum quantity.

IV. 7/12. 1 female spawned small amount 1/2 maximum. 4 males spawned, 1/2 maximum. 10 did not spawn.

V. 7/14. 6 females spawned, one heavy, 2 medium and 3 light. 9 males spawned, one heavy, 4 medium, 4 light. 10 did not spawn after hours.

Comment.—Light spawning activity compared with ten days ago which was remarkably heavy. Tendency to spawn reluctantly, some individuals spawning only after hours. One spawned after two hours, others slow to act. Only one good lot of eggs from twenty-five individuals, and none on July 12.

* *July 16, full moon.*

VI. 7/19. 4 females shed, 2 maximum, 2 light. 5 males shed, 3 maximum, 2 light. 6 did not spawn.

Comment.—Impression of light or medium spawning activity.

* *July 23, third quarter.*

VII. 7/25. 12 females shed, 5 maximum, 3 medium, 4 light. 7 males shed. Some very heavy, some light. 5 did not spawn.

Comment.—Impression of heavy spawning activity.

VIII. 7/31. 7 females shed, 4 heavy, one medium, 2 very light. 7 males shed, 5 heavy, 2 light. 2 did not spawn.

Comment.—Impression of heavy spawning activity, but not quite as heavy as at last new moon.

* *July 31, new moon.*

IX. 8/2. 5 females shed, 3 heavy, 2 light. 6 males shed, 4 heavy, 2 light. 7 did not spawn.

Comment.—Only one female spawned within an hour. And only two males within the first hour. (Why?) No others spawned for two and one half hours. Impression of poor spawning activity, reluctant and evident retention of eggs from some cause, also low percentage spawned after hours.

* *August 7, first quarter.*

X. 8/12. 11 females shed, 5 near maximum, 2 medium, 4 light. 14 males shed, all heavy or medium. 2 did not spawn within the first hour.

Comment.—This lot began to spawn after fifteen minutes, males more quickly than females. Most of the males began before thirty minutes. Some females began after twenty-five minutes but most of them after 40 minutes. Fourteen males and eight females had shed within one hour. Three females spawned after one and one half hours.

Impression of good heavy spawning activity and the reluctance observed a week ago was gone. The spawning was unusually prompt and vigorous. Cause?

* *August 14, full moon.*

XI. 8/16. 100 per cent. spawned. 6 females shed, 4 heavy, 2 medium. 3 males shed, 2 heavy, one medium. 1 spawned after 1½ hours heavily.

Comment.—Very heavy spawning activity. All except one spawned within an hour. Most of them began in 20 to 30 minutes. The last one spawned heavily after one and one half hours.

Heilbrun collected twelve Cumingia this date. All but one spawned.

XII. 8/20. 6 females spawned within an hour, 4 heavy, 2 medium. 13 males shed, 7 heavy, 3 medium, 3 light. 2 females spawned after 1½ hours. Very few eggs from these two. 1 did not spawn.

* *August 22, third quarter.*

XIII. 8/26. 100 per cent. spawned. 12 females shed during first hour, 5 heavy, 2 medium and 5 a mere trace. 9 males shed, 5 heavy, four light. 2 females spawned after one hour and forty minutes. The last two spawned very few eggs.

Comment.—Impression of heavy spawning activity during the past ten days. Five females gave only a very few eggs which indicates recent spawning on their part at the full moon period.

The inhibition to spawning noticed earlier is not now working. The heaviest spawning of the year has occurred during the past ten days.

XIV. 8/29. None spawned during the first hour. 4 females spawned a few eggs later, 1/10 maximum. 2 males shed a trace of sperm. 14 did not sperm.

XV. 8/30. 2 females spawned. Very few eggs, approximately 1/15 maximum. 3 males shed lightly, 1/10 maximum. 15 did not spawn.

* *September 1, new moon.*

XVI. 9/2. 5 females shed a few eggs, scarcely visible in the dish (a trace). 2 males made water slightly turbid with sperm. 18 did not spawn.

Comment.—Spawning negligible. Experiments for 1924 ended at this time. Experience has shown that spawning no doubt revived at the approach of the September full moon for a few days. This series of experiments shows that spawning was heavy early in July from the time the experiments of the season began until after the first quarter when they suddenly fell off not to be fully revived until somewhat after full moon. Then followed a week of heavy spawning until new moon.

Shortly after the first quarter in August the heaviest spawning of the year began and continued until the approach of the next new moon or from August 7 to August 29. Then came the usual late season, temporary cessation of spawning which presumably revived somewhat at the September full moon.

It is unfortunate that these experiments could not have begun by the middle of June and continued until the middle of September. The experience of several years leads one to believe that spawning began at full moon in June and reached maximum late in June. The variations in spawning activity under laboratory conditions show that the production of the gametes is greatest around full moon and least around the first quarter. These results are shown regularly except at the height of the breeding season when it may be masked by the rapidity of egg production.

SPAWNING EXPERIMENTS. 1926.

Cumingia tellinoides.

Table No. III.

The following table of experiments shows the duration of the spawning season and variations in spawning activity during the lunar cycle. From fifteen to twenty-five individuals were used in each experiment. They were isolated in stender dishes containing enough water to cover them fully. Spawning usually occurs within an hour. Collections were always made at low tide.

I. 3/1. No eggs and no spermatozoa.

II. 4/1. No eggs and no sperm.

III. 6/1. No eggs and no sperm. (Experiment by Hugh Montgomery.)

IV. 6/11. No eggs and no sperm. (Experiment by Hugh Montgomery.)

V. 6/15. No eggs, one male shed active spermatozoa, one fifth maximum.

* *June 18, first quarter.*

VI. 6/21. 2 females spawned, approximately 1/5 maximum. 2 males shed sperm, 1/4 maximum. 8 did not spawn.

Comment.—Eggs mature, cleavage normal. Spawning season opened between June 15 and June 20.

* *June 25, full moon.*

VII. 7/1. 6 females spawned, 1/4 to 1/3 maximum. 7 males shed sperm, 1/3 to 1/2 maximum. 2 did not spawn.

* *July 2, third quarter.*

VIII. 7/6. 9 females spawned rather abundantly, 1/2 to 3/4 maximum. 12 males shed sperm, 11 heavy, 1 light. 2 did not spawn.

* *July 9, new moon.*

IX. 7/15. 2 females spawned, 1/2 to 2/3 maximum. 12 males shed sperm, 10 medium, 2 light. 12 did not spawn.

Comment.—Light spawning, and many failed to spawn.

* *July 17, first quarter.*

X. 7/19. 2 females spawned 1/20 maximum (very few eggs). 4 males shed sperm, very light, 1/10 to 1/20 maximum, scarcely detectable in the dish. 13 did not spawn.

Comment.—Spawning all but extinct. Not even one fair lot of eggs obtained. Scarcely visible in the dish. Almost no spawning activity during the past week.

XI. 7/24. 13 females spawned lightly, 1/2 to 1/3 maximum. 7 males shed sperm, 6 heavy, 1 light. 2 did not spawn.

Comment.—Great revival of spawning but light in amount.

* *July 25, full moon.*

XII. 7/26. 8 females spawned, 4 heavy (maximum) and 4 medium (1/2 to 2/3 maximum). 12 males shed sperm, 6 heavy, 4 medium, and 2 light. 6 did not spawn.

Comment.—Very heavy spawning. The heaviest this year to date. All spawned promptly after thirty or forty minutes. Lunar periodicity is demonstrated clearly. Almost complete cessation of spawning ten days before full moon but came on again actively at full moon.

* *July 31, third quarter.*

XIII. 8/4. All spawned actively, 100 per cent.

* *August 8, new moon.*

XIV. 8/10. 6 females spawned, 3 heavy, 2 medium, 1 light. 11 males shed sperm, 10 heavy or medium, 1 light. 6 did not spawn.

XV. 8/13. 7 females spawned, 6 medium or heavy, 1 light. 7 males shed sperm, mostly heavy. 1 did not spawn.

XVI. 8/16. 100 per cent. spawned. 8 females spawned in quantity. 7 males shed sperm in quantity.

* *August 16, first quarter.*

XVII. 8/20. 5 females spawned (1/2 maximum or less). 7 males shed sperm, 1/2 maximum. 2 did not spawn.

XVIII. 8/21. 6 females spawned heavily, 1/2 to 3/4 maximum. 9 males shed sperm heavily. 5 did not spawn.

* *August 23, full moon.*

Comment.—There is no evidence of reduced spawning at the first quarter in August, whereas in July there was a clear-cut case.

XIX. 8/28. 7 females spawned, approximately 1/2 maximum. 12 males shed sperm, medium to light. 5 did not spawn.

Comment.—Spawning at this time by about 80 per cent. of the individuals but for the most part below the spawning of last week in quantity. Good lots of eggs still obtained, 1/2 to 1/3 maximum the rule. The quantity of eggs spawned by each individual is usually reduced toward the end of the season.

* *August 29, third quarter.*

XX. 8/31. 3 females spawned, 2 1/10 maximum, 1 1/20 maximum. 4 males shed sperm (1/4 maximum). 25 did not spawn.

XXI. 9/1. 6 females spawned 1/20 maximum (scarcely visible in the dish). 4 males shed sperm, very light, 1/10 maximum, water only slightly turbid. 14 did not spawn.

XXII. 9/3. No eggs and no sperm. Some extended the siphons and lashed them in characteristic manner but there were no eggs in the ducts.

Comment.—Very light spawning. Almost none since August 28. Spawning suddenly fell off, one week after full moon. Vigorous spawning continued for five days after full moon.

MARINE BIOLOGICAL LIBRARY WOODS HO MASS.

* *September 7, first quarter.*

XXIII. 9/9. 6 females spawned, 1 2/3 maximum, 4 1/5 maximum, 1 1/10 maximum. 5 males shed sperm, 3 1/2 maximum, 2 light. 19 did not spawn.

Comment.—Considerable revival of spawning noted. Much more active than last week, but still relatively insignificant. Good for so late in the season.

* *September 21, full moon.*

XXIV. 9/21. 3 females spawned, 1 maximum, 1 1/5 maximum, 1 1/10 maximum. 2 males shed sperm, 1 3/4 maximum, 1 1/2 maximum. 15 did not spawn.

Comment.—Revival of spawning evident, though not extensive. Quantities surprisingly large. Temperature 18° C., or 17½° C.

XXV. 9/10. Temperature 17° C. No eggs and no sperm.

Comment.—The spawning season of Cumingia ends between September 15 and October 1. This year spawning continued until the end of September. The foregoing data give no very definite information about the time of spawning in nature. It is evident that eggs are produced almost continuously although probably not uniformly. There is a lunar periodicity either in the production of the gametes or in the spawning or in both. Spawning by each individual occurs more than once during the season.

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CYTOLOGY OF SACCHAROMYCES CERVICIÆ WITH ESPECIAL REFERENCE TO NUCLEAR DIVISION.

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For some years following the discovery of cell division it was thought that direct division was responsible for the duplication of the nucleus, and even after the discovery of indirect division the former was still considered to be the usual method and mitosis was held to be a peculiar and infrequent phenomenon. Some held that the two processes were related and that mitosis was derived from the simpler and more primitive one, amitosis. However, as the investigation of this problem was continued evidence was gradually accumulated to show that mitosis is the usual if not universal method of cell division in higher animals and plants, and that amitosis is not a reproductive phenomenon at all, but has for its function the increase of nuclear surface in relation to volume and is rarely, if ever, followed by cytoplasmic division. This conception was well expressed by Conklin (1917) in the following passage, "Mitosis and amitosis are fundamentally unlike. Mitosis is the one and only method of bringing about equal division and distribution of the chromatic material of the nucleus. Amitosis is not a genuine divisional phenomenon at all, but merely a means of increasing the nuclear surface and of distributing nuclear material throughout the cell, comparable to nucleur lobulation, fragmentation or distribution. These two processes are not equivalent or even comparable, nor may one of them be converted into the other." Although this view is not universally held it is very generally accepted by cytologists at the present time.

The study of cell reproduction is much more difficult in Protozoa than in higher forms, as many of the types of intranuclear division have a superficial resemblance to amitosis. Minchin (1912) actually accredits the description of direct division in a number of protozoa, although he admits that some of the formerly supposed cases of amitosis have been shown to be merely simulations of it. Some of the authorities in this field, however, are willing to elevate the Protozoa to essentially the same position as Metozoa in this respect. Kofoid (1923) says: "In the first place amitosis as described in the Protozoa is either a pathological or degenerative process, as it is in the Metozoa, or it is based on a partial account of the normal process of mitosis in which the nuclear membrane remains intact throughout the whole process, as it does in the flagellates and rhizopods, and in its anaphases presents a superficial resemblance to pathological amitosis. The persistence of the nuclear membrane in no way interferes with the occurrence of chromosomes constant in number and kind. In other words the doctrine of chromosome continuity, in so far as amitosis is concerned, is no more affected in the Protozoa than in the Metazoa." The nuclear division (promitosis) of many of the lower protozoa is unquestionably very different from the mitotic division exhibited by higher plants and animals, but it seems likely that in all cases it is a simplified form of mitosis and is entirely unrelated to amitosis, even though it does superficially resemble it.

It seems as though the difficulty of studying nuclear division in any form is responsible for the persistence of investigators in describing it as amitotic. The tapeworm *Monezia* offers an example of this (Child, 1911). Yeast belongs in the same category, and we should realize the extreme difficulty of investigating this problem in yeasts by considering the minute size of the cells and the fact that for some time there was a heated controversy as to whether or not they even possess a nucleus. Some of those who took the affirmative, as has since been proved, were describing structures which belong to the cytoplasm. Wager (1898) and Wager and Peniston (1910) described as the nucleus, the actual nucleus, the vacuole, and a part of the metachromatic material surrounding the nucleus and vacuole. The division of this compound structure was described as amitotic in the case of budding and by an "intermediate step in karyokinesis" in the case of spore formation. This account of the indirect division of a cytoplasmic vacuole is comparable to the early figures of mitosis of the parabasal body (kinetonucleus) in trypanosomes and shows that without a favorable modification of technique the problem is almost invincible.

Some of the early contributors to our knowledge of the cytology of yeasts unquestionably saw and illustrated, with a fair degree of accuracy, not only the resting nucleus but also stages in its division. Janssens (1902) and Janssens and Leblanc (1898) considered the division of the nucleus to be an intermediate form of mitosis. Swellengrebel (1905) and Fuhrmann (1906) as a true mitotic process. Although these last two articles are in the main correct they have not been generally approved, and the ideas of Guilliermond (1904, '12, '17 and '19), which gain weight by the mere bulk of his work on yeast, seem to meet with more favor. This author, who is responsible for a large part of our knowledge of the well developed sexuality of yeasts and for an excellent account of the typical metazoan mitosis found in spore formation in *Schizosaccharomyces octosporus*, maintains that in bud formation the nucleus divides by a process identical with amitosis in the tissue cells of higher organisms, where, as Conklin asserts, it is not a reproductive phenomenon at all. To accept this would be to admit that mitosis and amitosis are fundamentally alike and interchangeable. This would undermine a large part of our knowledge of cytology and genetics. The problem most assuredly warrants critical study.

METHODS.

Pure cultures of *Saccharomyces cerviciæ* were used for this work. They were cultivated on both liquid and solid media. French proof broth was used for the liquid medium, French proof agar for the solid.

The organisms were transferred to slides which had been previously smeared with albumen fixative and the moist films were fixed either in corrosive-acetic-alcohol (95 per cent. alcohol saturated with mercuric chloride 95 parts, glacial acetic acid 5 parts) or Bouin's solution (saturated picric acid solution 75 parts, formalin 20 parts, glacial acetic acid 5 parts). Iron-alum-hæmatoxylin counterstained with light green or not counterstained at all was found to be the best means of staining. Delafield's hæmatoxylin and carbol fuchsin were tried without success, and eosin and orange G were found to be equally useless.

Fixation in corrosive-acetic-alcohol slightly shrinks the cells and gives the chromatic material in the cytoplasm such great affinity for basic stains that in most cases the nucleus is obscured. After fixation in Bouin's it does not stain so heavily and the nucleus can be seen in all cases, providing it is not too heavily stained. The successful technique finally developed was fixation in Bouin's and staining before the picric acid was completely washed out. This decreased the affinity of both nuclear and cytoplasmic chromatic material for stain, especially the latter. In this way the nucleus could be stained without showing the chromatic bodies in the cytoplasm at all. This procedure made possible a careful study of the nuclear chromatin both at rest and during division. Light green was found very valuable for the study of the metachromatic granules. Even when they are very abundant this stain will demonstrate their granular nature. In preparations in which no counterstain or the other acid stains are used they frequently appear as a single, large mass.

It was found that fixation and staining in carbol fuchsin followed by light green furnished an excellent method for demonstrating the alveolar nature of the cytoplasm. Although the nucleus could be seen in these cells its minute structure was not visible and the method is of no value for the present work. Light green is taken up by the gelatinous secretion sometimes given off by the yeast cells and consequently furnished a good means of demonstrating the presence of this substance.

Morphology.

Saccharomyces cerviciæ is a round to slightly oval yeast whose size ordinarily ranges from 5 to 10 micra. In old cultures the cells are occasionally elongated, sometimes crescentic (Fig. 7). The cell is enclosed in a thin though distinctly evident wall. This wall occasionally becomes greatly thickened and the cells pass into a very resistant stage (durable cells, chlamydospore) such cells can be kept dry for a year without destroying their viability (Figs. 10 to 13).

One of the most prominent structures inside the cell is the vacuole, small or entirely absent in very young cultures, but attaining a relatively great size after several days. In some cases it almost entirely fills the cell (Fig. 2). This vacuole is generally round, with a very regular outline. The cytoplasm is distinctly alveolar and contains numerous metachromatic granules. Occasionally cells are found with none of these granules, especially in very young cultures (Fig. 5) and sometimes there are only a few present, in which case they are located near the nucleus (Fig. 4). In old cells with a large vacuole there is frequently such a great mass of metachromatic material surrounding the nucleus that the latter body is obscured (Figs. 2 and 3), and the granular nature of the metachromatin is not discernible. Lines of fine granules can be seen leading from this mass to large granules in other parts of the cell, thus converting the metachromatic material into a connected unified system. These granular strands are especially evident where they go around the vacuole, and constitute the nuclear reticulum of Wager. Guilliermond figures basophilic granules within the vacuole. It is these granules above and below the vacuole which he sees and there are really no granules within the large vacuole. When material is fixed in Bouin's solution and stained in iron-alum-hæmatoxylin without washing out the picric acid the metachromatic granules do not stain. When this procedure is properly carried out, the well stained nucleus embedded in the alveolar cytoplasm is quite evident and easily studied. (Figs. 6 to 8.)

The nucleus of *Saccharomyces* is quite similar to that of higher plants, particularly *Phaseolus* (Kater, 1926). The nuclear membrane is equally as evident as in higher plants. Centrally located is a large basophilic nulceolus or karyosome from which radiate slender slightly basophilic linin strands. These strands run from the nucleolus to the nuclear membrane in identically the same manner as in *Phaseolus*. There are generally about six such strands visible. Just inside the nuclear membrane are located a number of chromatin granules. The larger ones are found at the points where the linin strands come in contact with the nuclear membrane. Except for the minute size of the nucleus (1 to 3 micra in diameter) it is hardly distinguishable from that of *Phaseolus*. A fruitful study of the finer points of the structure of the resting nucleus as well as following it through division would have been impossible without some means of staining it without affecting the metachromatin. It is this feature of our technique which makes the observations convincing. A centrosome could not be identified in the interkinetic cell nor could any variation in the cytoplasm surrounding the nucleus, comparable to sphere substance, be seen.

The yeast cell contains both fat and glycogen, the former usually in small globules in the vicinity of the nucleus.

It has been previously observed that yeast cells will give off a gelatinous secretion when permitted to dry gradually in a closed vessel. This is thought to play a part in the agglutination of yeast and the consequent clearing of the medium. In the present work it was found that dilution of the medium of an old culture with distilled water will produce this secretion (Fig. 9). The secretion has great affinity for light green.

Budding.

In young cultures the usual method of reproduction is by budding. The superficial features of this process are matters of common knowledge, and consequently, this description will be limited to the internal phenomena. The bulge in the cell wall and the entrance of cytoplasmic elements into the bud occurs some time before any change in the resting nucleus is observable (Fig. 14). The finely vacuolated cytoplasm is the first material to enter the bud. This is followed by the metachromatic granules. In those cells that contain a large vacuole the bud is generally formed near the nucleus and consequently near the greater part of the metachromatic material. The mass of this material near the bud separates into individually visible granules, part of which migrate through the isthmus into the bud, the rest moving to the opposite side of the parent cell (Figs. 15 and 21). Whether or not they divide at this time cannot be stated. Even after these granules are distributed to the two cells the nucleus is still in the resting condition. The large clear vacuole does not divide, but after the bud has attained almost the size of the parent cell a small vacuole appears within it and gradually enlarges.

After the bud has attained almost normal size and the distribution of metachromatic granules is completed the nucleus gives evidence of impending division. The chromatin accumulates on the linin strands, making them very evident and extremely basophilic. At the same time the disperse chromatin granules enlarge and the whole nucleus becomes a perfect miniature of an early prophase in *Phaseolus vulgaris* (Kater, 1926, Figs. 15 and 16). Meanwhile the nucleus remains stationary (Fig. 16). The steps in the transformation of such a nucleus into chromosomes cannot be followed with any degree of certainty, but many stages such as the one represented in Fig. 17 were seen. A knot of chromosomes probably occupying the old location of the nucleolus is here observed at the periphery of which individual chromosomes are becoming disentangled from the rest.

So far neither a dividing centrosome or spindle can be identified. However, as the chromosomes become arranged on the metaphase plate a spindle becomes visible (Fig. 18) and in some cases the ends are lodged in a granule perfectly comparable to the centriole in metazoa. No astral radiations are visible (Fig. 19). Such a centrosome has previously been reported in yeast (Swellengrebel, 1905; Guilliermond, 1917). In Fig. 18, the chromosomes are quite clearly separating in the same way as they do in higher plants and animals. Except for the difference in size the early anaphase represented in Fig. 19 could easily be mistaken for a metazoan mitotic figure. No careful attempt was made to count the chromosomes of *Saccharomyces* as their size makes it impractical. However, it seems certain that there are more than the four reported by Swellengrebel (1905) and Fuhrmann (1906). Probably at least twice that number.

The migration of the chromosomes through the isthmus into the bud is not at all clear. In some figures the orderly arrangement of the anaphase plates seems to be maintained (Fig. 22) while in others the chromosomes appear to enter the bud one at a time. In such cells the spindle disappears and only the irregularly arranged chromosomes can be seen (Fig. 21). In overstained slides specimens similar to the one illustrated in Fig. 22 very greatly resemble amitosis, and are probably responsible for the confusion on this point.

After being distributed to the two daughter cells the chromosomes become collected at one point and apparently fuse forming a uniformly basophilic mass in which the individual chromosomes cannot always be identified. The imbibition of achromatic material results in the alveolization of the outer part of this mass. The limiting membrane (nuclear membrane) is frequently bulged between the linin strands (Fig. 25). A continuation of alveolization results in a spherical nucleus with a nucleolus of moderate size, the resting condition.

The division of the cytoplasm in the isthmus occurs shortly after the separation of the chromosomes and the disappearance of the spindle (Fig. 24). Although the cytoplasm has separated, the cell wall frequently does not divide for a considerable length of time and holds the two cells together. In this way a number of cells are sometimes connected, somewhat resembling a mycelium (Fig. 8).

No attempt has been made to study the formation of ascospores, but according to previous accounts the nucleus divides by mitosis.

Discussion.

The above account leaves two points without adequate solution, namely the transformation of the nucleus into chromosomes and the migration of chromosomes through the isthmus. Fortunately these are matters of detail and the remainder of the account shows conclusively that the nucleus of yeast, in the formation of buds, does not divide by constriction, but that chromosomes are formed, divide (Fig. 18), separate, and give rise to daughter nuclei in the same way as in higher organisms, and yeast can be placed in the same category with higher animals and plants, and protozoa as enunciated by Conklin and Kofoid respectively.

To accept Guilliermond's descriptions of the indirect division of the nucleus in the formation of ascospores and direct division in budding would be to admit the reproductive nature of amitosis and, secondly, that a nucleus produced by this method may later divide, in the ascus, by perfectly normal mitosis. This would make untenable many of the generalized conceptions of the cytologist and the geneticist. It seems remarkable that the investigation of nuclear division in yeast has not previously attracted the attention of cytologists, as well as mycologists.

In the earlier work on *Phaseolus* it was found that the linin strands radiating from the nucleolus are actually the linin sheaths of chromosomes which persist through the resting condition and give the chromosomal vesicles morphological individuality during interkinesis. It seems probable that the linin strands of *Saccharomyces* are of the same nature. The collection of chromatin along them in the early prophase (Fig• 16) and the bulge between them in the telophase (Fig. 25) would indicate this. However, since the actual transformation of the prophase nucleus into chromosomes and the alveolization of early telophase chromosomes could not be followed with any degree of certainty a definite statement of chromosomal continuity in yeast cannot be made on a morphological basis as in *Phaseolus*, but the probable homology is certainly worthy of mention.

The existence of radiating linin strands alone would not justify this interpretation, since they are present in many protozoan nuclei where the nuclear membrane remains intact throughout mitosis. In such organisms the membrane is not a product of the linin sheaths of chromosomes, as in higher animals and plants, and the linin strands could not easily be interpreted in the same manner. Such a nucleus is found in Polytomella citri (Kater, 1925). In *Saccharomyces* the nuclear membrane disappears and consequently it is quite possible that the linin strands are homologous with those of *Phaseolus*.

Summary.

The nucleus of *Saccharomyces cerviciæ* divides by mitosis in the process of budding.

The chromosomes apparently form, divide, separate, and give rise to daughter nuclei in much the same way as in *Phaseolus*. The linin strands connecting the nucleolus and nuclear membrane probably represent sheaths of chromosomal vesicles.

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EXPLANATION OF PLATES.

Figures 1 to 5 and 15, 20, and 21 made from material fixed in corrosive-acetic-alcohol and stained with iron-alum-hæmatoxylin, Figs. 4, 5, and 15 counterstained with light green. The remaining figures from material fixed in Bouin's solution and stained as above. All drawings made with Abbe model camera lucida. Magnification 3,200 ×.

PLATE I.

FIG. 1. Small yeast cell showing fine granular lines of chromatic material in the cytoplasm, nucleus partly obscured.

FIG. 2. Nucleus entirely obscured. Granular lines above vacuole clear.

FIG. 3. End view of a cell similar to Fig. 2.

FIG. 4. Cell containing only a few metachromatic granules. Nucleus visible. Medium-sized vacuole.

FIG. 5. No metachromatic granules. Nucleus very large and structure clear.

FIG. 6. Nucleus above vacuole which is not so clear as in side view.

FIG. 7. Cell exhibiting crescentic form.

FIG. 8. Four connected cells showing manner of connection by unbroken wall.

FIG. 9. Cross hatching indicates gelatinous secretion which holds the cells together.

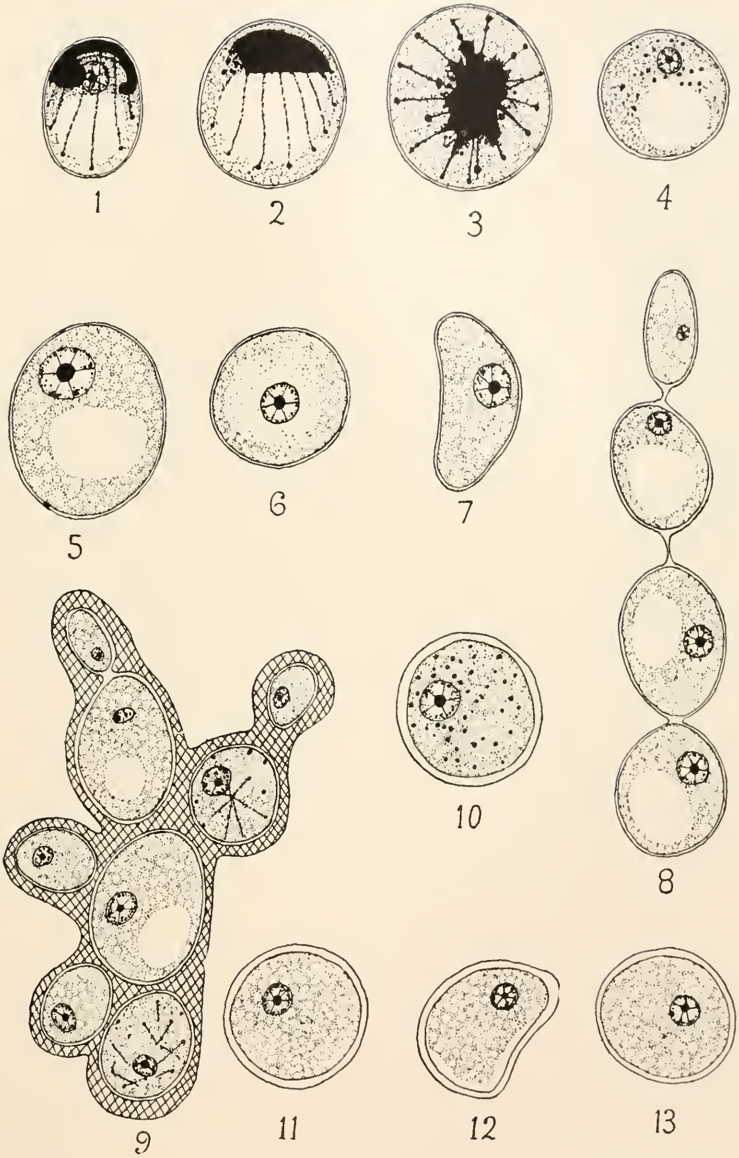
FIG. 10. Resistant cell. Note heavy wall and abundance of metachromatic granules.

FIG. 11. The same. Metachromatic granules disappeared.

FIG. 12. Resistant cell showing departure from spherical form and shrinking of protoplast.

FIG. 13. Resistant cell that has been kept dry for one year.

Drawings by G. T. Kline.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J. MCA. KATER.

PLATE II.

FIG. 14. Early bud. Extremely large vacuole.

FIG. 15. Metachromatic granules entering the bud. Nucleus not yet beginning division.

FIG. 16. Early prophase. The chromatin is collecting on the linin strands. A few metachromatic granules visible in both cells.

FIG. 17. The nucleus is breaking up into chromosomes. Slightly oblique view making bud appear abnormally small.

FIG. 18. Metaphase. The chromosomes are separating on the equator of the spindle.

FIG. 19. Anaphase. Centrosome and spindle very evident. Metachromatic granules in both cells.

FIG. 20. The same. Slightly later.

FIG. 21. The chromosomes are passing through the isthmus. A few metachromatic granules in opposite ends of both bud and parent cell. These are much smaller than the chromosomes.

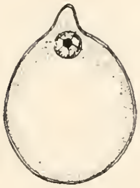
FIG. 22. Late anaphase. Spindle very evident. A second bud on the parent cell.

FIG. 23. The spindle not visible. The isthmus is closing. Chromosomes collected near one point.

FIG. 24. The cytoplasm has divided, the cells being held together by their walls. Chromosomes beginning the apparent fusion.

FIG. 25. Late telophase. Large nucleolus. Note bulges in nuclear membrane between linin strands.

Drawings by G. T. Kline.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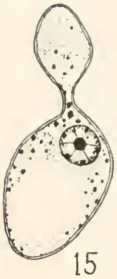
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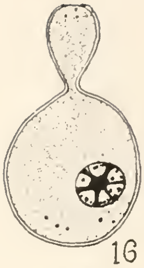
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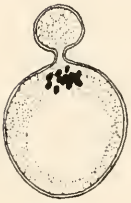
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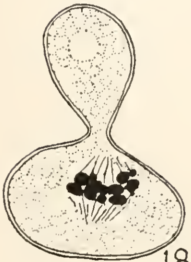
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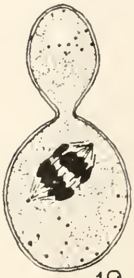
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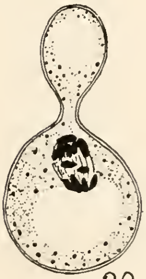
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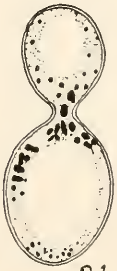
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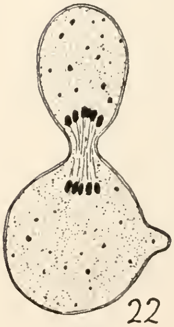
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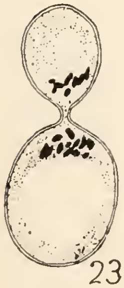
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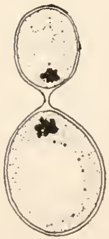
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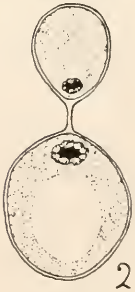
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J. MCA. KATER.

MEASURES OF INSECT COLD HARDINESS.

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Different measures of insect cold hardiness used by different workers may usually be reduced to the empirical survival test. Bachmetjew (1901) used the "vital temperature maximum" or the second time an insect reached the undercooling point. Duval and Portier (1922) considered that there was a freezing point below that ordinarily determined, the higher freezing point being that of the body fluids, the lower that of the body cells.

In strong contrast to the scarcity of measurements of insect cold hardiness, stand the many determinations by plant physiologists. Osmotic pressure as determined by freezing point lowering has been widely used from the time of Sachs and Pfeffer. Water content has been of value as a criterion of cold hardiness in plant groups far separated taxonomically. For example, Johnson (1923) used water content of peach buds as a measure of cold hardiness, and Steinbauer (1926) employed it for clover seeds. Newton and Gortner (1922) and Newton (1924) emphasize the importance of bound water to cold hardiness. Müller-Thurgau (1886) proved conclusively that some plants could survive freezing. The ability of a plant to survive freezing was defined by Harvey (1918) as cold hardiness.

The two kinds of insect cold hardiness (1) hardiness to the quantity factor of low temperature or ability to withstand long periods of relatively mild low temperature and (2) hardiness to the intensity factor of low temperature, or ability to withstand extremes of low temperature have been discussed in a previous paper. In the present paper cold hardiness to the intensity factor alone will be considered.

Closely associated with changes in cold hardiness are changes in moisture content. Insects dehydrated but not to the period of injury, can withstand temperatures far lower than undehydrated individuals. This is strikingly true for insects that are not self dehydrating to any large extent. Thus the Japanese beetle, *Popillia japonica* Newm., does not exhibit any marked body weight changes over winter when kept in moist surroundings, but can be experimentally dehydrated to half its body weight. When thus treated they are very cold resistant, having a survival temperature of as low as − 28° C. In contrast to the Japanese beetle larvæ there are some species of oak borers and caterpillars which are normally self dehydrating during the winter. The dehydration curves (Fig. 1) of *Isia isabella* Hy. Edw. and *Diacrisia virginica* Fabr. show a marked water loss as these caterpillars go into hibernation. At the period of inflection

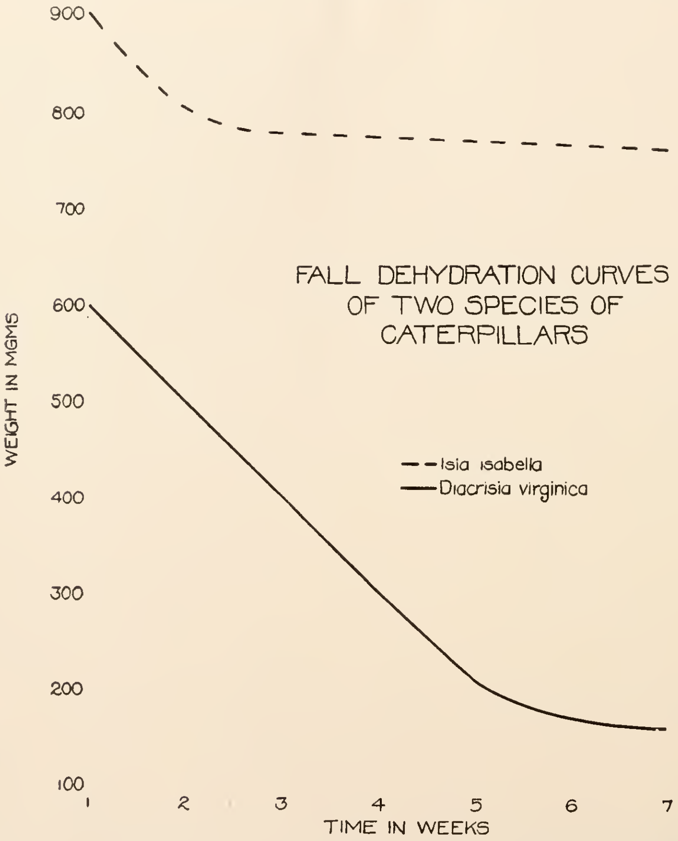

FIG. 1. Fall dehydration curves of two species of caterpillars, *Isia isabella* and *Diacrisia virginica*.

of the weight loss curve (Fig. 1) these insects can survive freezing. When the curve is plotted with rate against weight loss the point of inflection is brought out more clearly (Fig. 2). Up to the point of inflection of the weight loss curve the undercooling point of the blood is the minimum survival temperature. Beyond that point the undercooling point no longer measures the total cold hardiness which reaches to below − 40° C. There is no free body fluid on which a conductivity reading can be made.

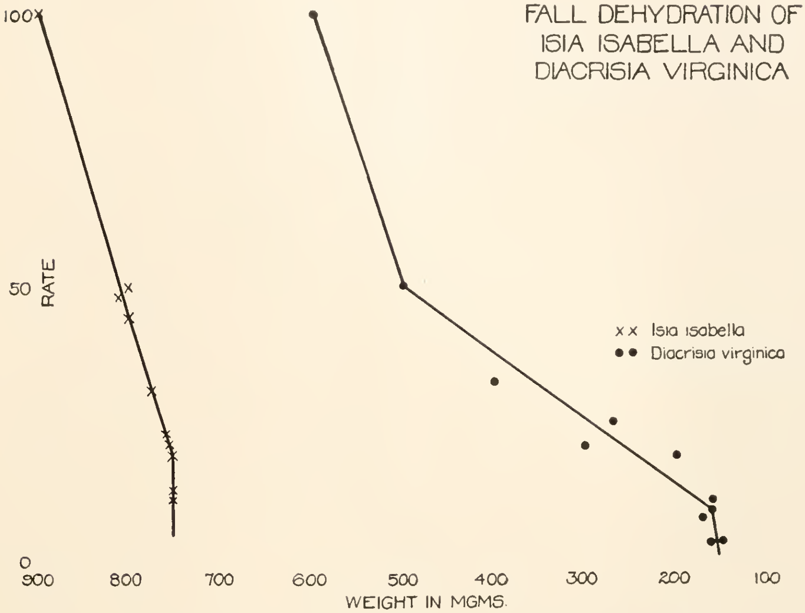

FIG. 2. Fall dehydiation of *Isia isabella* and *Diacrisia virginica*. $\frac{1}{\text{time}}$ or rate plotted against weight.

The oak-borers, *Synchroa punctata* Neum., *Dendroides canadensis* Lec., *Romaleum rufulum* Hald. also are normally self dehydrating but never to the extent of losing all their free water. Although very cold resistant, having survival temperatures of below − 40° C., at no time even in the deepest winter, is it impossible to obtain blood samples. But conductivity is found to be proportional to the survival temperature (Fig. 3). The water content of these insects, obtained by heating them in an oven for four hours at + 50° C., is only relative but does appear

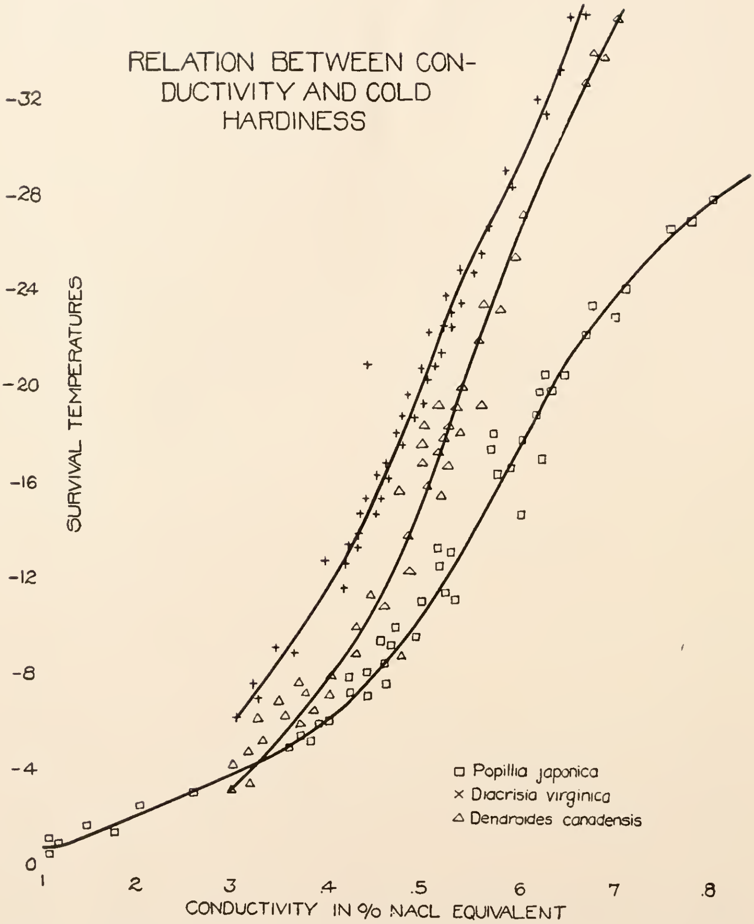

FIG. 3. Relation between conductivity and cold hardiness. *Popillia japonica* □, *Diacrisia virginica* ×, *Dendroides canadensis* △.

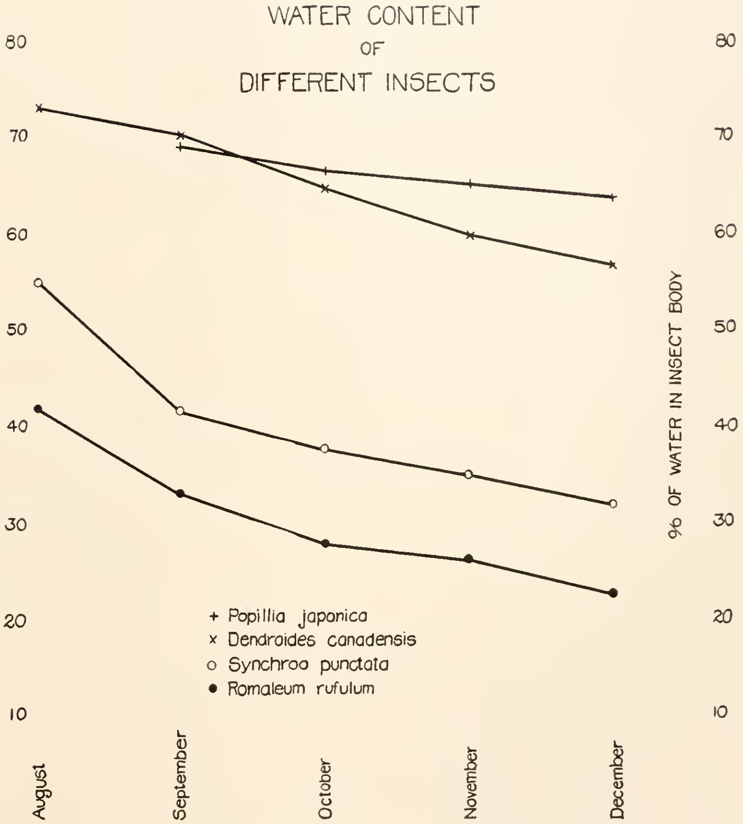

FIG. 4. Water content of different insects during the season at which they develop cold hardiness. *Popillia japonica* +, *Dendroides canadensis* ×, *Synchroa punctata* o, *Romaleum rufulum* ●.

to give comparable results with different species. The per cent. of water before and during hibernation of three species of oak-borers and of the Japanese beetle are shown in Fig. 4.

The Japanese beetle larvæ, *Popillia japonica* Neum. represent an ecological group far more protected than either the oak-borers or the woolly bear caterpillars. This species hibernates in the ground below the frost line. About 97 per cent. are third instar larvæ and about 3 per cent. second instar. There is a cyclic change in the cold hardiness of these larvæ, not as marked, however, as in the oak-borers but more apparent than in the

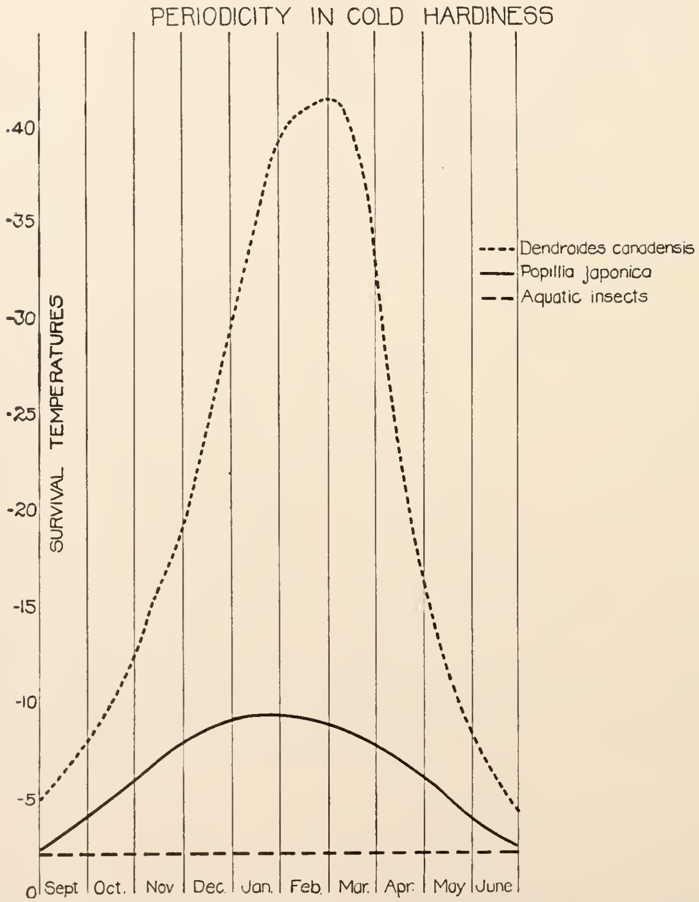

FIG. 5. Periodicity in cold hardiness. *Dendroides canadensis* – – –, *Popillia japonica* ——, aquatic insects — —.

aquatic insects where there is practically none, Payne (1926). This periodicity in cold hardiness is shown in Fig. 5. The relation between undercooling and survival temperatures is shown in Fig. 6. Cold hardiness greater than is usually found in their soil habitat can be induced in this insect by dehydration. Conductivity measurements of the blood of dehydrated insects were made. The greatest cold hardiness was found in the dehydrated insects and the least in those infected with wilt disease or

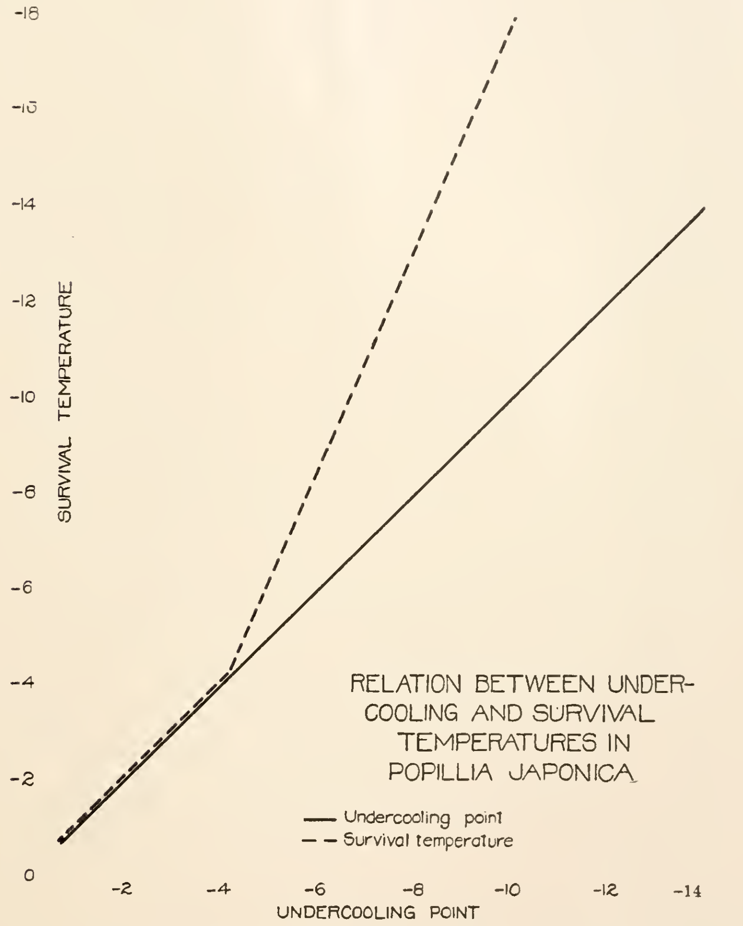

FIG. 6. Relation between undercooling and survival temperatures in *Popillia japonica*. Undercooling point ——; survival temperature — —.

polyhedrälskrankheit. In this disease both the freezing point and the conductivity of the blood approach that of water. In Fig. 3 the conductivities of the Japanese beetle larval blood are plotted against survival temperature. Cold hardiness in this species is more fully measured by conductivity than by either moisture content or undercooling point.

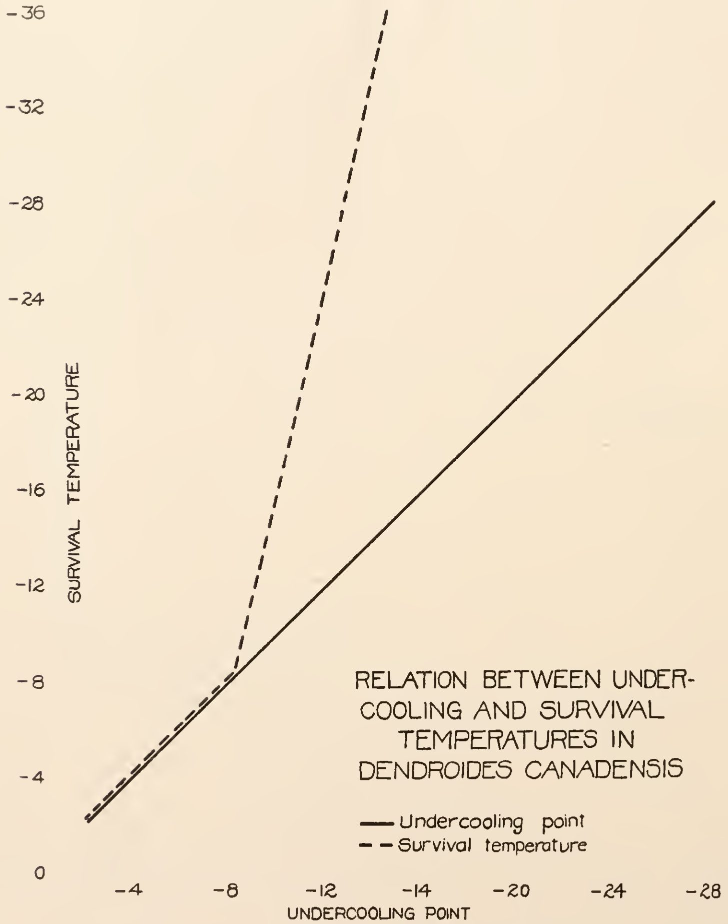

FIG. 7. Relation between undercooling and survival temperatures in *Dendroides canadensis*. Undercooling point ——; survival temperature — —.

SUMMARY.

1. Cold hardiness to the intensity factor of low temperature can be measured by moisture content, undercooling point, and blood conductivity.

2. Up to the time when a given insect can survive freezing, undercooling is a reliable measure of cold hardiness. Beyond the point when an insect can survive freezing, undercooling measures but a part of the total cold resistance of a given insect.

3. Conductivity measurements are found proportional to cold hardiness throughout the whole year. In some insects there is insufficient free body fluid in winter on which to determine blood conductivity.

4. For each species there is a different set of physical constants which measure the cold hardiness of that species.

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COMPENSATORY HYPERTROPHY OF THE TESTES IN BROWN LEGHORNS.[1]

L. V. DOMM AND MARY JUHN.

I. Introduction.

Compensatory hypertrophy of the surviving testis after unilateral castration was observed as early as 1890 by Ribbert (1). Ribbert worked on mammalian material, using young but almost mature rabbits. Ribbert removed the right or the left testis and then compared the surviving gland with controls of the same age after three months; considerable hypertrophy was almost always found. The hypertrophied testis weighed six times as much as one control gland in three cases. There was no difference in the degree of hypertrophy between the right or the left testis. If the operated animal did not increase in weight as much as did the normal controls, then the retained gland also showed a corresponding lack of development.

Lipschütz '22 (2) repeated these experiments of Ribbert, using also rabbit material for his experiments. It appears to follow from Lipschütz's paper that there is a compensatory increase in weight of the surviving testis after unilateral castration when the operation is performed on young rabbits. This increase in size and weight becomes progressively less the longer the operated animals are kept; about one year after the operation there is not much difference in the weight of the surviving testis and in that of one of the control pair of glands. Unilateral castration is not followed by a significant increase in the size of the remaining testis when the gonad is removed in adult rabbits. Lipschütz believes that his results indicate that the testis is incapable of true compensatory hypertrophy; the actual increase in weight observed being due only to a more rapid rate of growth of the isolated gonad.

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[1] From the Whitman Laboratory of Experimental Zoölogy of The University of Chicago. The expenses of this investigation were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

The two papers cited above report a certain difference in the results obtained. The discrepancy may be due to the time the surviving gonad was retained and also to the age of the animal at the time of operation.

We became interested in the problem of compensatory hypertrophy of the testis as applied to the material used in the laboratory for a variety of experiments, pure bred Brown Leghorn cocks. The experiments were begun early in July 1924 and terminated at the end of April 1925.

We were guided in outlining the course of the experiments by the following points of view: (*a*) the appearance of compensatory hypertrophy as such after the removal of one of the pair of gonads; (*b*) the influence of the age of the birds at the time of the operation on the possible increase in size of the surviving gonad; (*c*) whether the time that the surviving gonad is retained is of effect on the degree of possible hypertrophy taking place. (*d*) Finally we wished to determine if there was a significant difference in the amount of the hypertrophy taking place in the right or the left gonad after unilateral castration.

After the experiments were terminated and the data completed we became acquainted with a paper by Benoît '25 (3). This author carried out a series of unilateral castrations on White Leghorns; the operations were performed on three young birds of 18–20 days of age, on one young bird aged two months and on two birds aged seven months each. Control gonad weights were stated for the groups of different ages. According to the results obtained by Benoît, there is a very real increase in the weight of the surviving gonad when castration is carried out at an early date. In the three cases where unilateral castration was performed in baby chicks the surviving testes were retained for about twelve months. At this date each one of the hypertrophied testes weighed approximately as much as, or slightly more than, both testes of the control. The surviving gonad of the cockerel which was operated upon at two months was retained for seven months, at the end of this time it weighed almost 50 per cent. more than the control pair of testes, but it is important to observe that "controls" of this age vary greatly among themselves. Benoît observed no significant hypertrophy when unilateral castration was performed on birds aged seven months. The surviving testis was retained about a year. Benoît concludes that there is a hypertrophy of the surviving gonad when the one member of the pair is removed in very young birds; unilateral castration of older birds, after the testes have achieved approximately their normal size is not followed by a compensatory increase in the weight of the surviving gonad.

The results published by Benoît agree on the whole with those obtained in this laboratory, but we differ slightly from him in the observations on older birds as will appear in the discussion.

The phrase "compensatory hypertrophy" defines the conception, viz: that loss results in stimulating the growth of the surviving member to an extent that tends to restore a normal quantitative balance between the total gonad tissue and the bird. It involves the corollary that there is a normal quotient for weight of bird divided by weight of gonad tissue. The present study aims merely at testing this assumption. The difficulties arise from the fact that the assumed normal quotient of weight of bird divided by weight of gonad varies (1) with age very markedly; (2) with the time of year, age being the same; and that (3) no organ of the body probably is so susceptible to general conditions of health as the testis. These difficulties create numerous sources of error for any very exact formulation, so that we felt that it was not desirable in the present status of this subject with reference to our main problems to use a sufficient amount of material and time to reach quantitative results. The present study, although it gives positive results, is therefore merely suggestive.

It is a pleasure to express our thanks to Professor F. R. Lillie for his continued interest in the work and for his helpful suggestions during its course.

II. Data on Unilateral Castration.

All the unilateral castrations were carried out on pure bred Brown Leghorn cockerels that were obtained from one well-known source. The birds were divided into four groups, the first being about one week of age, the second sixteen weeks, the third twenty-four weeks and the fourth between thirty-two and forty weeks of age at the time of operation.

The operated birds and their controls in each group were hatched at the same time and kept in the laboratory under identical conditions. Comparisons were made only within the groups and in no case between birds of the same age but hatched at different periods.

The cockerels and their controls were weighed at the time of operation and the measurements of the head furnishings and spurs taken. These observations were repeated every eight weeks and a record kept of the condition of the experimental and control birds during the entire experimental period. The testes were removed through an incision between the last two ribs, the gland rapidly weighed and the volume obtained through displacement of normal saline. In the group of baby chicks where the testes were removed at about one week of age, the gonads were not weighed, but the length and width of the gland taken with a pair of fine callipers. The removed testes were fixed in Bouin's fluid at 37° C. and kept in the incubator at that temperature for several hours, varying with the size of the gonad. The usual procedure was followed in washing, etc., and the testes preserved in oil of wintergreen for future histological work.

The greater number of the chicks operated upon at one week of age was lost together with their controls owing to unfavourable weather conditions. The survivors were kept until they were thirty-two weeks of age and then completely caponized or killed.

In the three other groups the right gonad was removed from a certain number of cockerels and the left from a similar number. The surviving right or left testis was then retained for eight, sixteen and twenty-four weeks respectively, at the end of each of these periods one cockerel having a right testis, one cockerel having a left testis and two control birds were completely caponized or killed.

We found in the course of our observations that loss of weight on the part of the cock is reflected in a corresponding diminution of the size of the testes.

The data obtained are outlined in the tables given below. The age of the bird at the time of the operation, the weight of the bird at the beginning and end of the experimental period is stated. The weights of the removed, hypertrophied and control glands are expressed in grammes and in per cent. of the body weight of the fowl.

In the group A (Table I.), where unilateral castrations were performed on baby chicks aged one week, six operated birds and five controls survived for a period of about thirty-one weeks. Unilateral castrations were performed on thirty-five baby chicks and there were a large number of controls; the mortality was due not so much to operative effects but to the very unfavourable weather conditions. Of the six surviving operated birds, three had a left testis while the other three had a right one. Each surviving left gonad is larger than any of the ten control testes; in two of the three cases each surviving left testis is larger than

TABLE I.

RESULTS OF UNILATERAL CASTRATION AT ONE WEEK.

No.	Observations at Time of Operation.			Weighings at 32 Weeks.		
	Chick, Weight.	Removed Testis.		Bird.	Surviving Testis.	
		Length.	Width.		Weight.	Per Cent. Weight.
53	45.40	R. 3.5	2.0	1,460.80	L. 19.95	1.35
56	54.50	L. 4.1	1.2	1,507.50	R. 7.35	0.48
44	67.15	R. 4.5	2.0	1,545.85	L. 13.00	0.83
49	66.00	L. 5.0	1.3	992.25	R. 0.85	0.095
46	57.95	R. 5.5	1.5	1,275.85	L. 18.70	1.46
50	50.90	L. 6.0	1.0	1,048.96	R. 5.10	0.49
62c[1]	45.00			1,020.60	R. 0.65	0.063
					L. 0.55	0.053
64c	46.50			1,602.55	R. 12.80	0.79
					L. 11.25	0.70
65c	47.65			1,219.05	R. 6.95	0.57
					L. 7.50	0.61
63c	43.00			1,489.15	R. 8.20	0.55
					L. 7.75	0.52
66c	44.00			994.25	R. 6.20	0.61
					L. 5.85	0.58

The measurements given for the testes removed at unilateral castration are in mm. All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

any of the five control pairs with one slight exception (No.64). These results cannot be due to chance; we are therefore justified in concluding that a surviving left testis, the partner of which is removed at one week after hatching, exhibits a much greater amount of growth than it would have done, approaching in some cases twice the normal growth.

Preliminary histological examinations of sections of hypertrophied testes indicate that all the gonad tissues are equally concerned in this increase in size.

Table II.

Results of Unilateral Castration at 16 Weeks.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 24 Weeks.	
					Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
819......	992.25	R. 0.20	0.020	1,687.60	L. 9.60	0.56
820......	1,077.35	L. 0.35	0.031	1,630.90	R. 6.35	0.38
826c[1]....	1,105.65			1,630.90	R. 8.40	0.50
					L. 9.80	0.60
830c.....	1,162.35			1,602.55	R. 3.00	0.18
					L. 3.10	0.19
					At 32 Weeks.	
815......	1,048.95	R. 0.36	0.033	1,687.60	L. 30.00	1.81
823......	907.20	L. 0.17	0.018	1,247.40	R. 15.82	1.26
829c.....	1,460.80			2,071.10	R. 14.11	0.66
					L. 15.78	0.75
831c.....	1,048.95			1,857.70	R. 11.82	0.60
					L. 14.11	0.75
					At 40 Weeks.	
824......	1,048.95	L. 0.65	0.061	1,574.20	R. 20.18	1.28
825......	963.90	L. 0.19	0.19	1,517.50	R. 20.25	1.31
833c.....	992.25			1,574.20	R. 11.61	0.73
					L. 14.30	0.90

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

The three surviving right testes on the other hand showed no such increase in size, and in fact did not differ significantly from a single control testis.

The second group of young cockerels was castrated at sixteen weeks and the results are tabulated in Table II. The surviving testes were retained eight, sixteen and twenty-four weeks respectively. It was originally planned to observe the degree of compensatory hypertrophy to forty-eight weeks at which time the birds have been fully mature for about sixteen weeks. However a number of birds died owing to one cause and another so that the last data were obtained on cocks aged forty weeks and only on two right testes.

There is no observable compensatory hypertrophy either of the right or the left testis when the glands were retained only for eight weeks after the operation. There was an increase in size during this period but this was identical with the control glands. After a period of sixteen weeks, however, both the left and the right surviving testes show a considerable degree of compensatory hypertrophy, the left testis being heavier than both testes together of each of the two control pairs; the right testis on the other hand, while it exhibited a high percentage weight, was only slightly heavier absolutely than a single control testis. Three birds were available for observation after twenty-four weeks, two of them having a right testis each while the third served as control. Each of the two right testes weighed about 75 per cent. as much as the control pair, the degree of compensatory hypertrophy on a percentage basis being similar to the amount observed after sixteen weeks.

Table III. gives the data for the next group. The cockerels in this group were unilaterally castrated at twenty-four weeks and then observed to forty-eight weeks at intervals of eight, sixteen and twenty-four weeks, respectively. After eight weeks the surviving left testis showed a certain degree of hypertrophy, weighing much more than one of the control pair of gonads and only slightly less than the other. The right testis had not increased at all as compared with the normal. The same is true after sixteen weeks; the left surviving gland is larger than any one testis of the control pairs but not as heavy as one of the

TABLE III.

RESULTS OF UNILATERAL CASTRATION AT 24 WEEKS.

No.	Weighings at Time of Operation.			Later Observations.		
	Bird.	Removed Testis.		Bird.	At 32 Weeks.	
					Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
810....	1,574.20	R. 0.45	0.027	1,801.00	L. 10.90	0.60
801....	1,332.45	L. 0.20	0.016	1,715.95	R. 0.65	0.041
901c[1]....				1,574.20	R. 1.50	0.095
					L. 1.31	0.093
902c....				2,241.20	R. 6.65	0.29
					L. 8.95	0.39
					At 40 Weeks.	
813.....	1,020.60	R. 0.25	0.024	2,127.80	L. 17.05	0.80
804.....	1,545.85	L. 5.0	0.32	2,241.20	R. 8.60	0.37
809c....				2,212.85	R. 12.70	0.57
					L. 14.60	0.65
907c....				2,099.45	R. 7.70	0.36
					L. 8.0	0.38
					At 48 Weeks.	
808[2]....	963.90	R. 0.20	0.020	1,162.35	L. 3.18	0.27
812....	1,573.20	L. 2.70	0.17	1,212.85	R. 10.83	0.83
904c....				2,127.80	R. 13.23	0.62
					L. 11.81	0.55
905c....				2,042.75	R. 4.15	0.20
					L. 3.45	0.11

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

[2] No. 808 was ill during the first half of the experimental period and lost considerable weight which it had not regained at the time the bird was killed and the surviving testis removed.

pairs, while the right surviving testis is not as heavy as control single testes. After twenty-four weeks there was no hypertrophy at all to be observed in the left surviving testis; as this bird had been in poor condition and lost considerable weight during the experimental period, we do not attach much significance to this case. The right surviving testis is probably to be regarded as hypertrophied, being heavier than one of the control pairs and its percentage weight greater than any one testis of the other control pair.

In the group where the cocks were mature (Table IV.), there was not so much difference to be observed in the hypertrophy of the surviving testes. The left testis had increased relatively more in size compared with the gland removed at the operation than the right testis. The hypertrophying testes were retained for only eight weeks, the observation period being planned to extend only to forty-eight weeks of age. The left surviving testis was larger than any single testis of the two control pairs, but not as heavy as either pair together. The right surviving testis was heavier than one control pair and almost equal to the other pair, being 0.86 per cent. of the body weight while the two control testes pairs weighed 0.78 per cent. and 0.97 per cent. respectively. While the cases are few, the evidence seems to indicate compensatory hypertrophy in this group also after a very short period.

TABLE IV.

RESULTS OF UNILATERAL CASTRATION AT 32–40 WEEKS.

No.	Weighings at Time of Operation.			Weighings at 40–48 Weeks.		
	Bird.	Removed Testis.		Bird.	Surviving Testis.	
		Weight.	Per Cent. Weight.		Weight.	Per Cent. Weight.
121......	1,574.20	R. 3.95	0.25	2,042.75	L. 13.87	0.67
114......	1,517.50	L. 6.55	0.43	1,659.25	R. 14.27	0.86
123c[1]....	1,517.50			1,517.50	R. 7.36	0.48
					L. 7.52	0.49
128c.....	1,829.35			1,687.60	R. 5.0	0.39
					L. 4.93	0.39

All weights are in grammes. Per cent. weight = percentage of weight of testis to total weight of bird.

[1] c = control.

II*a*. SUMMARY OF RESULTS OF UNILATERAL CASTRATION AT VARIOUS AGES.

The preceding tables and descriptions demonstrate that removal of one of the testes pair induces an increase in size of the retained partner when the retention period is of sufficient length, with the exception of the right isolated testes of young chicks (Table I.).

When unilateral castration is performed on young cockerels the left surviving gonad hypertrophies to a greater degree than does the right one, the one differing result obtained in No. 808 being due to the bird's general condition.

The increase in weight of the isolated testis becomes manifest only after a certain interval following castration, the interval being apparently somewhat longer for the right hypertrophying testis than for the left one when younger birds are operated upon.

TABLE V.

DATA COMPILED FROM TABLES I.–IV. CONTROL TESTES WEIGHTS STATED AS AVERAGES.

Age of Bird.		Weight of Isolated Testis.	Average Weight of Controls.	
At Operation.	At Removal.		Testis Pair.	Single Gland.
One week........	32 weeks	L. 13.00 L. 18.70 L. 19.95 R. 0.85 R. 5.10 R. 7.35	13.54 (5p.)	6.77
16 weeks........	24 weeks	L. 9.60 R. 6.35	12.15 (2p.)	6.07
	32 "	L. 30.00 R. 15.82	27.91 (2p.)	13.98
	40 "	R. 20.18 R. 20.25	25.91 (1p.)	12.85
24 weeks........	32 weeks	L. 10.90 R. 0.65	9.10 (2p.)	4.80
	40 "	L. 17.05 R. 8.60	21.50 (2p.)	10.75
	48 "	L. 3.18 R. 10.83	16.32 (2p.)	8.16
32–40 weeks.....	40–48 weeks	L. 13.87 R. 14.27	12.40 (2p.)	6.20

All weights are in grammes.

The latent period is much shorter when adult birds are unilaterally castrated and here the right isolated testis hypertrophies at the same rate and in the single case available even to a slightly greater degree than the left (Table IV.).

Comparison with the averages of the controls rather than with single controls as set forth in the tables increases the probability of these conclusions as examination of Table V. shows. Individual cases emphasize these general conclusions (Table I., Nos. 53, 44, 46. Table II., Nos. 815, 824, 845. Table IV., Nos. 114, 121).

Lipschütz's suggestion for mammals that unilateral castration produces only a more rapid rate of growth rather than a definitive compensatory enlargement of the surviving gonad does not seem a probable interpretation of our results. It would mean that there would be no actual plus in weight of the isolated gonad over one of the control testes pair at the end of the developmental period of the glands. Such a statement requires a definition of the endpoint of growth of the testes, and in view of the normal variation in the weight of the testes as well as the seasonal variation the feasibility of such an absolute determination appears questionable.

The cocks are mature at thirty-two weeks and compensatory hypertrophy as defined is demonstrated not only at this time but as late as forty-eight weeks.

III. Normal Size Relations of Right and Left Testes.

During the course of the experiments we accumulated some data on the size of the right and the left testes of normal cocks. The majority of the observations were made on the gonads of different birds but the records of the control birds in the preceding tables are for pairs. Tables VI. and VII. give the measurements obtained in one week old chicks. The length and width of the right and left testes are given as it was impracticable to secure accurate weights. The weights of the chicks are also stated for comparison. There appears to be a very slight advantage in size on the part of the left testes at this age. In older birds we find such an individual variation occurring in birds of the same age and even of approximately

TABLE VI.

MEASUREMENTS OF LEFT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
51	4.4 mm.	1.5 mm.	45.15 gs.
61	5.1 "	2.0 "	55.30 "
62	4.0 "	1.8 "	45.0 "
0	3.5 "	1.1 "	39.30 "
63	5.0 "	1.2 "	43.0 "
64	4.5 "	2.0 "	46.50 "
65	3.0 "	2.0 "	47.65 "
56	4.1 "	1.2 "	54.50 "
64A	4.0 "	1.1 "	40.40 "
66	4.0 "	1.5 "	44.0 "
67	5.0 "	1.5 "	49.50 "
68	5.0 "	1.9 "	44.75 "
69	4.8 "	1.9 "	49.90 "
50	6.0 "	1.0 "	50.90 "
49	5.0 "	1.3 "	66.0 "
48	5.3 "	1.3 "	57.85 "
40	4.5 "	1.5 "	52.80 "
47	5.2 "	1.7 "	57.85 "
39	5.0 "	1.3 "	63.80 "

No. of Cases.	Average of Measurements of Testes.	
	Length.	Width.
19	4.6 mm.	1.5 mm.

TABLE VII.

MEASUREMENTS OF RIGHT TESTES REMOVED FROM CHICKS AGED ONE WEEK.

No.	Testes.		Weight of Chick.
	Length.	Width.	
52	3.5 mm.	1.0 mm.	43.75 gs.
54	4.3 "	1.1 "	43.87 "
53	3.5 "	2.0 "	45.40 "
55	3.0 "	1.5 "	42.60 "
57	5.0 "	1.5 "	49.0 "
58	4.0 "	1.0 "	43.30 "
59	4.0 "	1.3 "	45.60 "
61	5.1 "	2.0 "	55.30 "
70	4.0 "	1.5 "	39.90 "
46	5.5 "	1.5 "	57.95 "
44	4.5 "	2.0 "	67.15 "
43	6.7 "	1.5 "	56.30 "
42	6.0 "	1.5 "	51.10 "
41	5.0 "	1.5 "	59.0 "

No. of Cases.	Average of Measurements of Testes.	
	Length.	Width.
15	4.5 mm.	1.2 mm.

identical weight, that valid conclusions cannot be drawn from the data obtained in different cocks. The tables compiled are omitted for this reason.

Where testes of one pair were observed as was done for the control cockerels (Tables II.–IV.) the left testes were larger than the right in one case out of two at twenty-four weeks. At thirty-two weeks, the left testes were larger in two out of three pairs, while at forty-eight weeks, the left testis was very slightly heavier (0.01 per cent.) than the right one in one pair; it was smaller than the right testis in two pairs and finally there was one pair in which the gonad weighed exactly the same amount on the left and on the right side.

The tendency of the left testis to be rather larger than the right one in embryonic chicks has been observed by a number of authors. Firket, '14 (4), states that the right testis is noticeably smaller than the left one in the chick at the seventh day of incubation and quotes Semon, '87 (5), as saying that the left testis is much larger at the beginning of its development.

According to Swift, '16 (6), the left embryonic testis is noticeably larger than the right one in the five day chick and the germinal epithelium of the left gonad is also thicker and more extensive. This difference in favor of the left testis is also visible in the six and nine day chick. Riddle, '16 (7), finds no difference between the right and the left testes in common fowl, the age of the birds is not stated.

The greater tendency towards hypertrophy of the left testis discussed under II.*a*, is presumably associated with this embryonic condition, and is of interest in comparison with the very pronounced asymmetry of the female.

IV. Discussion.

From the results described in the preceding pages as well as from the experimental data published by Benoît, the occurrence of compensatory hypertrophy following unilateral castration in young male fowls seems to be well established. We found compensatory hypertrophy of the retained gonad also in adult cocks, differing in this point from Benoît's observations. The period during which the surviving testes were permitted to hypertrophy was shorter in our cases than in those reported by Benoît and it is possible that the explanation for the different results obtained may be found in this fact.

The problem of compensatory hypertrophy of the gonad presents its teleological as well as its physiological aspects. The term itself has teleological implications; from this point of view the "purpose" might be either to provide increased reproductive capacity, which appears unnecessary, or to establish a balance of hormones. There again the solution is unsatisfactory for less than one testis is sufficient for maintenance of sex characters as shown by Pézard, '21 (8); '25 (9); Champy, '25 (10).

Physiologically considered it would appear to be obvious that the growth of testis tissue is balanced against something else in the organism.

The general bodily metabolism favors the growth of a definite amount of gonad tissue and no more. The removal of one testis of a pair leaves a balance of conditions favorable to the continued growth beyond its normal size of the surviving member which thereupon responds in proportion to its growth capacity up to the limits of the favorable metabolism. When unilateral castration is performed very early this may result in a single testis greater in weight than a normal pair (Table I., case 16, Table II., case 815), whether there may be a progressive limitation of capacity for compensatory growth with increasing age as maintained by Benoît is still an open question as far as our own results are concerned.

No theory is put forth in explanation of the change in the reaction between gonad and organism which follows removal of one of the gonad pair; it is shown by the facts. But the importance of the principle appears again in the transformations of the female following ovariotomy. The right rudimentary gonad responds with a proliferation of the kind of tissue of which it is composed at the time the demand on it is created, thus producing the various types of right compensatory growth described in completely and incompletely castrated hens by Domm, '24 (11); '27 (12). The principle of compensatory hypertrophy is also illustrated in the growth of grafts.

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V. Summary.

1. Unilateral castration in Brown Leghorn cockerels is followed by compensatory hypertrophy of either the right or the left retained testis when the operation is performed on birds aged 16, 24 and 32–40 weeks.

2. The removal of the right or the left testis in chicks aged one week caused a compensatory hypertrophy of the left retained gonads only, after a period of thirty-one weeks, in our experiments (Table I.).

3. There may be a certain period before increase in weight of the retained gonad over the controls becomes manifest; the length of this period is variable.

4. The left retained testis shows a greater tendency towards hypertrophy than does the right.

5. There seems to be a difference in the weight of the left and the right normal testes; this is in favor of the left gonad in very young birds and then gradually seems to become shifted to the right testis as the bird becomes older and reaches maturity.

6. A tentative suggestion is made, that there may be some relation between the greater amount of germinal epithelium in embryonic left testes and the greater tendency towards hypertrophy of the left surviving gonad which is particularly manifest when unilateral castration is performed on young birds.

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NOTE ON THE HEMOLYTIC ACTION OF SEBRIGHT SERUM ON LEGHORN CORPUSCLES.[1]

MARY JUHN.

In a series of experiments on grafts of Leghorn testes into Sebright capons and Sebright testes into Leghorn capons carried out by Mr. Roxas in this laboratory (1), the observations were made that the Leghorn testis takes readily in the Sebright but that the inverse is not the case. A much greater mortality was also observed in the Leghorn hosts having Sebright grafts than in the Sebrights having Leghorn grafts. In actual figures:

	Leghorn Testis into Sebrights.	Sebright Testis into Leghorns.
No. of birds	38	50
Died from known causes	12, 31%	8, 6%
Died, cause unknown	8, 21%	24, 48%
Survived to end of exp.	18, 47%	18, 36%
Birds with succ. grafts (% of surviving birds)	11, 60%	3, 8%

According to a verbal communication by Mr. Roxas, the Leghorn capons with Sebright grafts that succumbed, showed evidences of pronounced anæmia, this being evident in the lack of color of the headfurnishings which became progressively almost a dead white.

In attempting to determine the causes for the greater mortality among the Leghorn hosts as well as the reason for the much smaller per cent. of successful grafts of Sebright testes into the Leghorns, the following suggestions present themselves. Considering first the conditions in the Sebrights having Leghorn testes grafts, the per cent. of takes is high; the mortality which may be attributed to the graft (cause of death unknown, 21 per cent.) is relatively not so pronounced. We may believe then that the Leghorn tissues are readily incorporated in the

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[1] From the Whitman Laboratory of Experimental Zoölogy of the University of Chicago. The expenses of these investigations were supported in part by the Committee for Research in Problems of Sex of the National Research Council; grant administered by F. R. Lillie.

Sebright organism, they soon become vascularized, and being supplied with nutritive substances, persist and even show active growth. There is no extended necrosis in the Leghorn grafts beyond some presumably occurring when it is first implanted and previous to vascularization.

When Sebright testes are implanted into Leghorn tissues however, there is a relatively high percentage of mortality attributable to the graft (cause of death unknown, 48 per cent.) and a very low percentage of takes, 8 per cent. of the surviving birds and only 6 per cent. of all the birds operated upon. There is no evidence of vascularization of the Sebright testis grafts, on the contrary the appearance of the Leghorn capons after Sebright implantations have been made lead one to believe in a greater activity of the lymphocytes and a subsequent elimination of the destroyed graft tissues into the blood stream of the host. Blood counts before and after grafting as well as control of the body temperature would presumably serve to determine the accuracy of these assumptions. The resorption of the graft in a large percentage of cases with subsequent mortality of the host from one cause or another is however apparent.

There is an extensive literature on the subject of the toxicity of organ extracts when injected intraperitoneally, subcutaneously or intravenously. The intravenous injections produce the most rapid lethal effects, but subcutaneous injections of organ paste into guinea pigs, were reported by Brieger and Ulenhuth (2) to kill the animals within 24 hours after the injection. This effect was correlated by Dold and Kodama (3) with the toxic action of tissues in a state of destruction and with the causes of death after burns. Pfeiffer (4) states that in acute cases of death from burns, the cause of the mortality may be traced to toxic poisoning induced by protein fission products. These products appear in excessive quantities owing to the resorptive destruction of the proteins which have been changed and killed through heat.

It appears reasonable in view of the findings in comparable fields reported above, to correlate the percentage of deaths in the Leghorns having Sebright grafts with the non-success of these grafts. The continuous resorption of the graft tissues, may, and probably does, set free into the blood stream of the host toxic substances resulting from the destruction of the implant, and these toxic substances finally prove fatal to the carrier of the graft.

The point of interest is the reason for the different results obtained in these cross-transplantations. The possibility of some specific differences in the blood of the two breeds of fowl was suggested by Prof. F. R. Lillie; the tests made to establish the presence of such differences are described in the latter part of the paper.

My thanks are due to Professor Lillie both for suggesting the problem reported here and for his continued helpful interest in the work.

Some experiments carried out by Sokoloff (5) serve to illustrate that such conditions can be found in other vertebrates. Sokoloff working on homotransplantations in rabbits found the presence of three types of blood and states that grafts only take when made into animals of identical blood constitution with the donor. When grafts are made into animals where the serum of the host agglutinates the corpuscles of the donor, the graft shows complete necrosis. This destruction of the graft cells leads to the production of specific antibodies in the host which have an unfavorable action on the graft. Any graft will cause the formation of some percentage of antibodies but these are counteracted by the graft as soon as vascularization is established and they then disappear from the blood stream.

Furthermore according to Sokoloff, immunization by intraperitoneal injections of an emulsion of the organ to be grafted causes the appearance of antibodies as well, and the presence of these antibodies inhibits take and growth of the graft.

In studying the conditions in the Leghorns and Sebrights, no tests were made for the determination of circulating antibodies. The sera of Leghorn and Sebright cocks and capons were tested for their agglutinating and hemolytic action on the corpuscles of all four kinds of birds.

The experiments were repeated four times, the preparation of the serum and the corpuscle suspension being identical in every case. For the corpuscles blood was drawn from the ventricle into a syringe moistened with a 1.5 per cent. sodium citrate solution, 1 cc. of blood was injected into 19 cc. of 1.5 per cent. sodium citrate, the corpuscles then washed four times, centrifuging at low speed to just sediment the corpuscles and the final suspension being brought to 5 per cent. in normal saline. For the serum, blood was run into small test tubes and kept at room temperature over night.

The serum was diluted for all the experiments 1 : 4 with normal saline; the corpuscle suspension being 5 per cent. as stated above. Agglutination tests were made in the hanging drop according to the method outlined by Ascoli (6). No agglutination was observed in any of the serum-corpuscle combinations.

In the tests for the possible hemolytic action of the sera of any one of the four birds, sixteen hemolysis tubes were used in each experiment. Four of the tubes were controls, having the own corpuscles added to the serum, the other twelve were all the possible combinations. One cc. of the 5 per cent. corpuscle suspension was rapidly run into 1.5 cc. of the serum dilution. The tubes were then shaken, placed in the incubator at 38.5° C. for two hours and shaken again every quarter of an hour during this interval. The tubes were then placed in the ice-box at + 9° C. over night and observed the following morning. Furthermore a 5 per cent. corpuscle suspension in saline was always preserved to the end of the period of observation.

The table given demonstrates the results obtained more clearly than any written description. The greater tendency of the Sebright cock and capon serum to hemolyze the Leghorn corpuscles may be noted, but attention must be drawn to the fact, that this is not a constant phenomenon and that in the case of the Sebright capon serum the own corpuscles are hemolyzed to approximately the same degree.

In a single experiment not recorded in the table, the sera of all the birds was diluted 1 : 40 with normal saline. At this degree of dilution distinct hemolysis was observed in the tube having Sebright capon serum and Leghorn capon corpuscles. There was not even a trace of hemolytic action to be noted for any of the other serum-corpuscle combinations.

The different action of the sera tested does not prove an actual difference in the tissues but it is of interest to note the

SERUM.

	Sebright ♂.				Sebright Capon.				Leghorn ♂.				Leghorn Capon.			
Corpusc.	I	2	3	4	I	2	3	4	I	2	3	4	I	2	3	4
Sebr. ♂	–	+?	–	–	++	o	++	++	+?	+?	–	+	+?	+?	–	–
Sebr. c.	o	o	–	–	+++	o	++	++	+	+?	–	++	+	++	–	–
Legh. ♂	+++	+++	–	–	++	o	+	++	+?	–	–	–	+?	+?	–	–
Legh. c.	+?	+?	–	–	+?	o	++	+++	+	+?	–	+	–	+?	–	–

I, 2, 3, 4 are the four separate experiments referred to in the text.

o = no tube. – = no hemolysis. +? = very faint traces of hemolysis. + = hemolysis distinct. ++ = hemolysis definite. +++ = entire tube hemolyzed.

parallel between the hemolytic action of some of the Sebright sera on Leghorn corpuscles and the non-success of Sebright grafts in Leghorns except in a very small percentage of cases.

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CONFIGURATIONS OF BIVALENTS OF HYACINTHUS WITH REGARD TO SEGMENTAL INTERCHANGE.

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Introduction.

In the majority of the flowering plants examined by the writer, and apparently also in most of those investigated in this respect by others, the homologous chromosomes, which form bivalents at the reduction metaphase, are joined only at the extreme ends. As examples, Canna and Datura may serve, in which this rule holds in the triploids as well as in the diploids. In the largest bivalent of *Uvularia*, however (Belling, 1926), there are additional points of junction (nodes) not at the ends. The short and medium chromosomes of *Uvularia* seem usually to be connected at or near the constriction, and the same is the case with the short and medium chromosomes of *Hyacinthus* (Belling, 1925). These will not be further considered here.

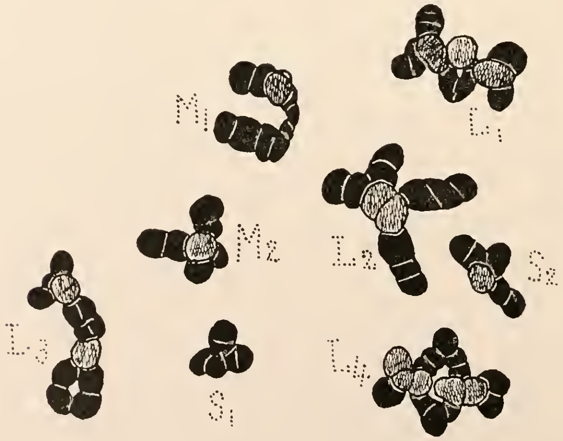

Fig. 1. Camera drawing of the eight bivalents of the diploid hyacinth, squeezed from a pollen-mother-cell. The four large bivalents are alone considered here. They are described in the text.

But the four large bivalents of *Hyacinthus* show, like the large bivalent of *Uvularia*, many connections not at the ends (Fig. 1). It has been pointed out in regard to *Uvularia* (Belling, 1926) that the simplest hypothesis is that these connections (nodes) not at the ends represent places where two of the four chromatids have undergone segmental interchange by fracture and recombination. In *Hyacinthus* it can apparently sometimes be seen with the microscope that two of the four chromatids are bent back at a node, so as to continue along the same sides of the bivalent (Fig. 3). It has also been shown that the homologous chromosomes of the rings and V's formed by the large bivalent of *Uvularia*, acted when separating as if they were not merely twisted across one another, but had undergone a process which produced some interlacing of chromatids at the nodes. This would prevent the simple untwisting of the homologues at the anaphase, and such untwisting has been shown not to occur in *Uvularia* (Belling, 1926). This would also lead (as has been abundantly shown by Janssens, 1924, and others, in animals) to the separation of whole upper and lower halves of vertical rings and V's, and to one chromatid passing up and one down from both sides of horizontal rings or V's, which may get smaller as the process advances, without opening up. This is what takes place in *Uvularia*, and apparently also in *Hyacinthus*.

In *Hyacinthus*, as already stated, the homologues are not always connected at one or both of the extreme ends, but are connected at other places (nodes). This is especially the case with the four long chromosomes. Hence a study of these may show, by the nature of their configurations and their mode of separation at the reduction metaphase, whether the nodes correspond to what would be expected if they were due to segmental interchange between chromosomes (crossing-over of genes).

If the nodes in the long bivalents of the hyacinth are due to segmental interchange, the following phenomena should be observable: (1) the nodes should occur at *different points* in the bivalents in different cases; (2) these nodes should be at equal distances from the ends of both homologues; (3) the nodes should be visible at the late prophase (diakinesis stage or earlier) as well as at the metaphase; (4) the horizontal rings or V's should split into separate chromatids, while the vertical rings or V's should divide into upper and lower halves, without separating into chromatids; (5) the numbers of bivalents with one or two nodes should permit of a calculation of the numbers of chromatids with segmental interchange at no, one, or two points, which should possibly more or less resemble the occurrence of no, single, or double crossing-over in *Drosophila*. In such a calculation, the numbers of chromatids with no segmental interchange would be equal to twice the cases of single nodes plus the number of cases of double nodes. The total number of chromatids with one point of interchange would be got by adding twice the number of bivalents with single nodes to twice the number with two nodes. While the chromatids with two points of interchange are equal to the number of bivalents with two nodes. It should be possible to test these five points.

(It seems obvious that a junction of homologous chromosomes at the ends has no relation with segmental interchange. It is probably otherwise with junctions at the point of constriction of the chromosome, where segmental interchange may well take place.)

It was for the purpose of testing this hypothesis that the present study was made.

Large Bivalents of *Hyacinthus*.

The variety of *Hyacinthus orientalis* investigated was one of those formerly studied (Belling, 1925), and was chosen because it could be readily identified by the flowers, and had marked characters even in the bulbs. This was the diploid clone called "Yellow Hammer." The bulbs were obtained in October, and put into water during that month and the next. Division of the pollen-mother-cells usually accompanied the development of the first roots. The pollen-mother-cells were instantaneously fixed by being squeezed out from the anthers into iron-acetocarmine. The chromosomes were observed with Zeiss' water-immersion objective 70, yellow-green light, and a water-immersion condenser.

At the first metaphase in the pollen-mother-cells (Fig. 1) the four long chromosome pairs commonly assume one of six different configurations (Diagram I.). Three of these are shown in Fig. 1, where the cross (L_2) is near the center, two single rings with double V's are on the right (L_1 and L_4), and a double ring and V on the left (L_3). Four forms in the late prophase are shown in Fig. 2. They are: (*a*) the ring and V; (*b*) the double ring and V; (*c*) the ring and two V's; and (*d*) the figure of 8. The cross and the triple ring were not found free, the bivalents being usually clustered at this stage. Several configurations are drawn in Fig. 3. It may be specially noticed that the four examples of the cross shown in Fig. 3 have the junction in different positions with regard to the chromosome ends. The cross in the middle

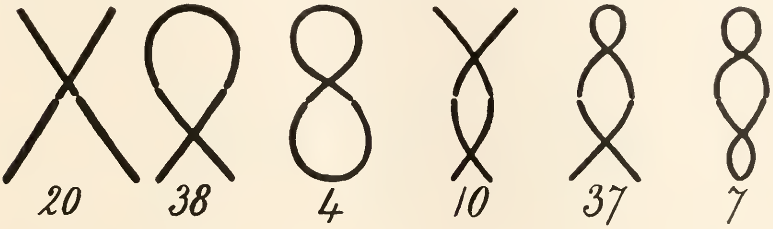

DIAGRAM 1. Numbers of different configurations of large bivalents. The cross, single ring and V, and figure of 8, have one node; while the ring and two V's, the double ring and V, and the triple ring have 2 nodes.

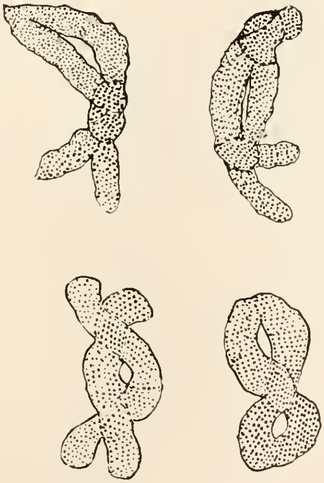

FIG. 2. Four camera drawings of large bivalents at the late prophase. (This stage is difficult to obtain.)

has arms which taper to the center, the two homologues having apparently joined at the constrictions. The last two drawings in the lowest line of Fig. 3 are the same bivalent at two different focusses, apparently showing one transverse and one reflexed

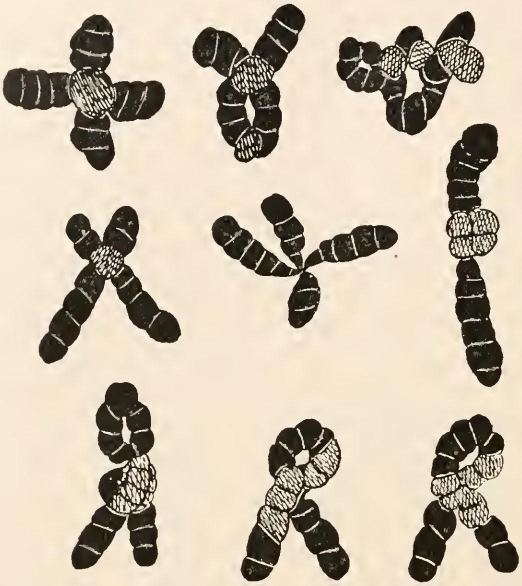

Fig. 3. Camera drawings of eight bivalents. The four crosses have the nodes at different points, but always at equal distances from the ends of the homologues. The lowest arm of the second cross in the second line is much foreshortened. The last two figures show the effects of change of focus on the node.

chromatid of one homologue, the same thing being often observable at a different focus in the other homologue also. These are pulled out by the spindle fibers which are attached at the apex of the bend at the median constriction.

One hundred and sixteen of the long bivalents were classified in six groups (Diagram 1), mostly after squeezing chromosomes and cytoplasm from the cell. The results were (Diagram 1): 38 cases of the ring and V; 20 X's; and 4 figures of 8 (totalling 62 with one node); 37 cases of the double ring and V; 10 cases of the ring and two V's; and 7 cases of a triple ring; totalling 54 cases with two nodes. No bivalents with triple nodes were certainly demonstrated. If only two chromatids underwent segmental interchange at any one node, as seems to be indicated by the microscopical phenomena, we have for the resulting pollen grains and any particular long chromosome, on the hypothesis of segmental interchange: no segmental interchange (124 + 54), 178; single point of interchange (124 + 108), 232; and double interchange, 54; out of a total of 464 chromosomes or pollen grains.

This gives in percentages: chromosomes with no interchange 38 per cent.; chromosomes with single interchange, 50 per cent.; and chromosomes with double interchange, 12 per cent. The ratio of single to double interchange on the hypothesis is thus 4.3 to 1.

This is not far from the numbers of no, single and double points of crossing-over given for the first chromosome of *Drosophila melanogaster* by Morgan (1925). The second and third chromosomes of *Drosophila*, however, seem to differ in this respect.

In Figs. 4 and 5, there are examples of the separation of chromatids and homologues which tend somewhat to prove the hypothesis of segmental interchange. In Fig. 4, L_1 is especially instructive. Here on one side of the constriction (and spindle fiber attachment) there was apparently a horizontal ring, and

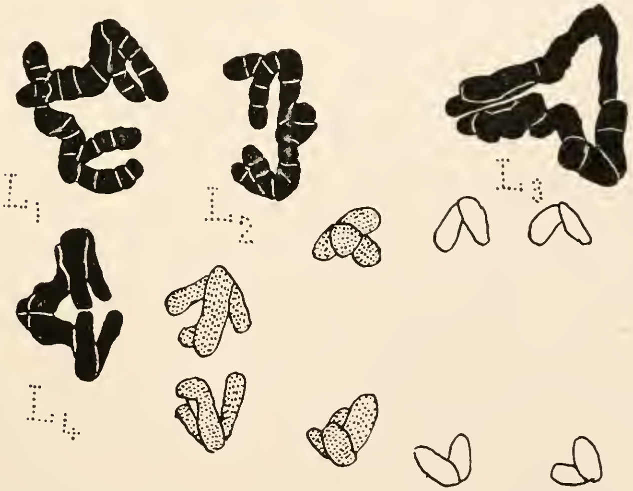

FIG. 4. Camera drawing of the bivalents in a cell in which the four large ones had not completely separated, the drawing paper being shifted after each was drawn. Some are foreshortened, especially L_2 and L_4.

on the other side perhaps a vertical ring and a small V; or only a large vertical V. The horizontal ring evidently split into two ring chromatids, while the vertical ring remains. In L_3 the constriction is in the vertical ring. (L_2 and L_4 show nothing more.) In Fig. 5, L_1 shows apparently the separation of the parts of a cross, the two horizontal members of which can be seen splitting into chromatids, one passing to each pole.

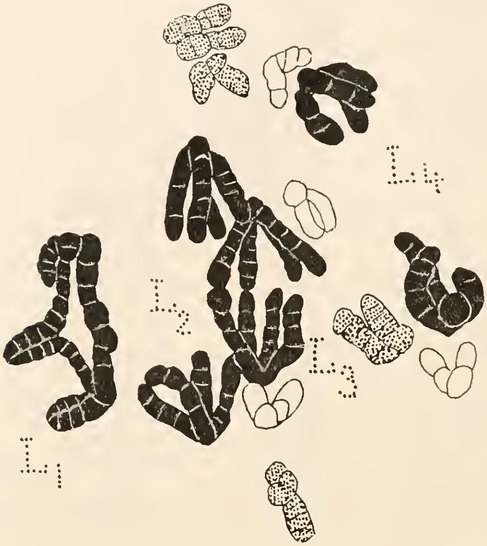

FIG. 5. Slightly more advanced stage than Fig. 4.

DISCUSSION.

The following points seem most worthy of notice.

(1) At some nodes it appears as if both chromatids of each homologue could be seen, one obliquely transverse, and the other reflexed. The spindle fiber is often attached (at or) near the node also at the median constriction.

(2) Some rather scanty data seem to show in *Hyacinthus*, what is more abundantly demonstrated in *Uvularia*, namely, that single chromatids of each homologue pass to the poles from both sides of horizontal rings and V's, and that the two chromatids of each homologue remain connected in the separate halves of vertical rings and V's. So the horizontal rings and

V's diminish in size as their chromatids are pulled out by the spindle fibers towards the poles.

(3) The numbers of cases of possible segmental interchange in the chromatids of the large bivalents, calculated from the numbers of nodes, agrees roughly with the numbers of cases of crossing-over found in the first chromosome of *Drosophila*, which is especially favorable for this study.

Some process of segmental interchange seems demanded by the genetic evidence in *Drosophila*, *Zea*, *Lathyrus*, and the other plants and animals which have shown cases of crossing-over. Hence it is apparently the natural scientific procedure to accept segmental interchange as a working hypothesis to account for the nodes and internodes of the chromosome pairs in the *Orthoptera* and other animals, and also in *Uvularia* and *Hyacinthus*. This is the more imperative in that there seems no other available working hypothesis.

SUMMARY.

(1) The four large bivalents of *Hyacinthus* show in 62 cases one node, and in 54 cases two nodes where the homologues cross.

(2) At these nodes it can apparently be seen with the microscope that one chromatid of each homologue passes obliquely across, while the other seems bent back along the other homologue.

(3) The hypothesis of previous segmental interchange at such a point is assumed until a better hypothesis is found.

(4) The numbers of chromatids showing such points of segmental interchange, according to the hypothesis, calculated from the 116 bivalents examined, were 38 per cent. with no interchange, 50 per cent. with one point of interchange, and 12 per cent. with two points of interchange.

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